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TITLE: Enhanced Androgen Signaling with Androgen Receptor Overexpression in the Osteoblast Lineage Controls Skeletal Turnover, Matrix Quality, and Bone Architecture

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14. ABSTRACT

Androgens have been shown to be important mediators of bone growth and remodeling independent of estrogen. A better understanding of the consequences of androgen action in bone is particularly important given increased anabolic steroid abuse. In addition, since bone architecture and bone material properties play important roles in stress fracture, analysis of this model represents a unique opportunity to characterize the consequences of androgen action in both genders on bone microarchitectural quality and the integrity of the skeleton *in vivo*. We genetically engineered transgenic mice in which androgen receptor (AR) overexpression is skeletally targeted to better understand the role of androgen signaling in bone. The central hypothesis of this proposal is that AR transactivation in the osteoblast lineage provides key regulatory signals that regulate the progression of osteoblast differentiation and osteogenesis, control the resorption of calcified bone, and modulate lineage determination in the marrow, to influence skeletal architecture and matrix quality. In the first year, we have successfully created the second line of AR-transgenic mice using the col2.3 promoter to drive AR expression. Ongoing analysis of studies (proposed in Specific Aim 1) suggest that AR transactivation (in young adults of col2.3 with col3.6 ARtransgenic animals) in mature osteoblasts is primarily responsible for mediating the effects of androgen on matrix quality and/or mineralization, while immature cells mediate effects of androgen on the periosteum and potentially body composition. Further, enhanced androgen signaling during growth appears to produce a low turnover state and may be detrimental to skeletal quality with more damageability. Preliminary analysis of studies (proposed in Specific Aim 3) has shown androgenmediated enhancement of apoptosis of cells in the osteoblast lineage *in vivo* and *in vitro*. Combined these results raise concerns regarding androgen administration in the still-growing skeleton. Studies in adults are ongoing.

15. SUBJECT TERMS
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Introduction

Androgen deficiency (as a result of aging, hypogonadism, glucocorticoid therapy, or alcoholism), and other behaviors (chronic smoking, malabsorption and bone marrow malignancies) are associated with the development of osteoporosis in men (1). Osteoporosis is also an important and debilitating side effect of androgen deprivation therapy in conjunction with the treatment of prostate cancer (2, 3). At any one time, osteoporosis affects 20 million Americans. Nearly one-quarter of the patients who suffer a hip fracture die within the first year; 50% of patients are unable to walk without assistance; and 33% are totally dependent (4, 5). Of the 1.3 million bone fractures that can be attributed to osteoporosis every year, 150,000 are hip fractures that occur in men with lifetime risk for the development of fracture at nearly 15% (6). In addition, it is also clear that androgens have an important but very much under-appreciated role in women (7). Other health problems may also be affected by androgen action, including atherosclerotic vascular disease, age-related weakness and disability, memory loss, etc. Since osteoporosis is often coupled with a hypogonadal state, developing an understanding of androgen action in the skeleton may provide insight into development of novel therapeutics for the treatment of osteoporosis and metabolic bone disease.

The distinct contribution of androgen to the maintenance of a healthy skeleton remains controversial, since the major androgen metabolite testosterone can serve as the substrate for the production estradiol via aromatase activity. As a consequence, some testosterone action may result from estrogen receptor-dependent activation after conversion to 17-ß estradiol. Overexpression of androgen receptor (AR), combined with the use of non-aromatizable androgens that cannot serve as a substrate for aromatase conversion (e.g. 5α-dihydrotestosterone; DHT), should enhance our understanding of the *specific* role for androgen in bone biology. The goal of this program is to gain a comprehensive understanding of the cascade of molecular and cellular events by which androgen signaling influences skeleton homeostasis.

Our proposed studies have substantial military significance. The stated goals of the Bone Health and Military Readiness program are to advance the understanding of methods to improve bone health of young men and women, to enhance military readiness by reducing the incidence of fracture during physically intensive training, and to reduce the incidence of osteoporosis later in life. As little is known about the direct actions of androgens on osteoblasts, our comprehensive approach using unique animal models of enhanced androgen responsiveness with distinct bone-targeted AR-transgenic families, combined with the novel studies of DHT modulation of osteoblast differentiation and osteoblast-osteoclast signaling, will provide insights into normal bone homeostasis. Understanding the consequences of androgen action in bone is particularly important given increased anabolic steroid abuse. In addition, since bone architecture and bone material properties play important roles in stress fracture, analysis of this model represents a unique opportunity to characterize the consequences of androgen action in both genders on bone microarchitectural quality and the integrity of the skeleton *in vivo*.

Body

In the first year of this grant, we have focused primarily on the creation and characterization of a distinct set of AR-transgenic mice, col2.3 AR-transgenic families, by employing a smaller collagen promoter fragment to drive AR overexpression in mature osteoblasts (as outlined in Specific Aim 1). Thus, in addition to the col3.6 AR-transgenic families we have previously created with AR overexpression in stromal cells and throughout the osteoblast lineage including mature osteoblasts, the col2.3 AR-transgenic mice now also provide models for the characterization of enhanced androgen signaling in distinct skeletal compartments. Importantly

in both models, enhanced androgen action occurs only in those cells with elevated levels of AR (skeletally-targeted) as a consequence of enhanced AR signaling, without changes in circulating steroid levels and without systemic androgen administration. Because of distinct and overlapping expression profiles, comparison of the skeletal phenotypes characterized in these two models of enhanced androgen action are postulated to aid in the identification of cells within the osteoblast lineage that are most important in mediating a specific response in bone modeling/remodeling characteristics. For example, phenotypes identified as similar in both col2.3 and col3.6 suggests that mature osteoblasts/osteocytes are important mediators of the response, since there is overlap in promoter activity in those cell types. In contrast, phenotypes that are more pronounced in col3.6 than col2.3 suggest that stromal or immature osteoblasts are primary mediators.

Our goal as proposed in Specific Aim 1 is to contrast the skeletal phenotype of col2.3 with col3.6 AR-transgenic animals (with different AR overexpression profiles in the osteoblast lineage), in the adult and in the hypogonadal state in both genders, to identify direct androgen actions *in vivo*. This analysis will allow us to test the hypothesis that distinct profiles of AR overexpression in the osteoblast lineage will result in distinct skeletal phenotypes between col2.3 vs. col3.6 AR-transgenic mice.
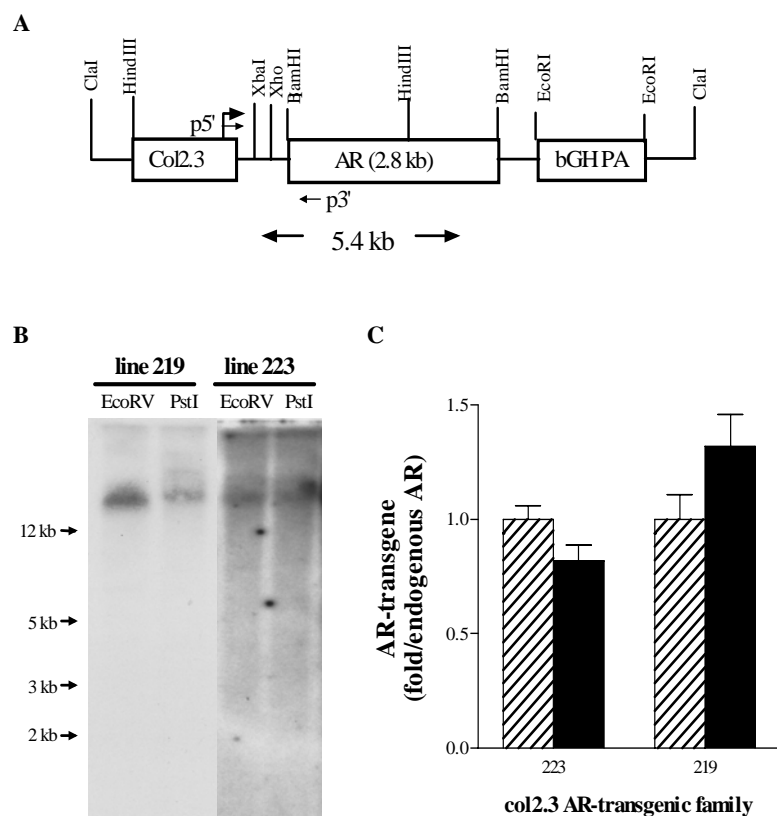
Fig. 1. Generation of col2.3 AR-transgenic mice. **A**. Schematic representation of the col2.3 AR-transgene. **B**. Southern analysis for characterization of insertion sites. Genomic DNA was isolated form both col2.3 AR-transgenic founder lines (223 and 219), digested with *Eco*RV or *Sal*I and subjected so standard Southern blot analysis after hybridization with the colAR transgene product. Analysis indicates a single insertion site. **C**. Copy number relative to the endogenous AR gene (~one copy for both lines) was estimated by real-time PCR analysis of genomic DNA. All data is presented as mean ± SEM.

Generation of col2.3 AR transgenic mice: We have recently derived a distinct set of AR-transgenic mice using a shorter fragment of the collagen promoter, the col2.3 promoter, which controls AR-transgene expression only in mature osteoblasts. The full length rat AR cDNA fragment was cloned downstream of the rat 2.3-kb collagen I α1 promoter (col2.3) and upstream of the bovine growth hormone polyadenylation (bGH-PA) signal (Fig. 1A). The 5.4-kb linear DNA fragment containing the col2.3AR fusion gene was excised from the flanking vector with *Cla*I and used for pronuclear injection of mouse B6D2F1 embryos to generate the col2.3 AR-transgenic founders. Positive founders were identified by PCR genotyping. For analysis, two independent col2.3 AR-

transgenic lines are being bred to wild type B6D2F1 mice to control for so-called position effects of the inserted transgene (family 223 and 219). Southern analysis has demonstrated a single site of integration for both families using the PCR fragment as a probe (Fig. 1B). AR-transgene copy number relative to the endogenous AR gene was estimated by real-time PCR analysis using genomic DNA. Results demonstrate roughly one copy of the col2.3 AR-transgene in each line (Fig. 1C).

Table 1. Real-time qRT-PCR analysis of col2.3 AR-transgene mRNA expression in tissues from col2.3 AR-tg mice

Tissue	Col2.3 AR-tg level	Fold difference vs. calvaria
Calvaria	1.0000 ± 0.2373	n/a
Tibia	0.0307 ± 0.0098	-32.57
Thymus	0.0066 ± 0.0011	-151.52
Lung	0.0054 ± 0.0005	-185.19
Heart	0.0047 ± 0.0019	-212.77
Kidney	0.0044 ± 0.0006	-227.27
Fat	0.0027 ± 0.0007	-370.37
Spleen	0.0025 ± 0.0004	-400.00
Muscle	0.0006 ± 0.0000	-1666.67
Skin	0.0006 ± 0.0002	-1666.67
Ear	0.0004 ± 0.0001	-2500.00
Liver	0.0004 ± 0.0002	-2500.00
Tendon	0.0003 ± 0.0000	-3333.33
Intestine	0.0000 ± 0.0000	n/d

To further characterize col2.3 AR-transgenic mice, we determined the pattern of AR-transgene mRNA expression in a wide variety of tissues by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Tissue was harvested from male col2.3 AR-transgenic mice, total RNA was isolated and gene expression was characterized by qRT-PCR analysis as we previously described (8). As shown in Table 1, AR-transgene expression under the control of the col2.3 promoter results in bone-targeted expression, consistent with other reports (9) and as we have also seen with col3.6 expression patterns (10).
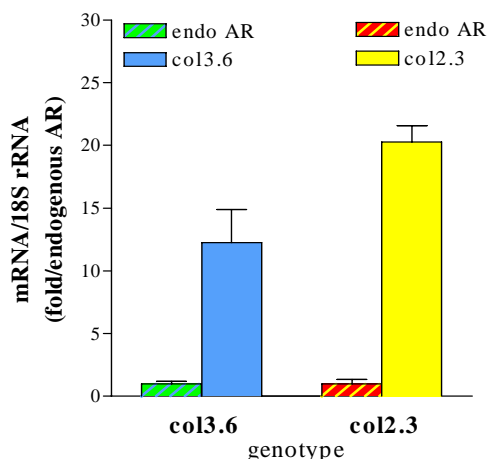

Fig. 2. Expression of AR transgene in calvaria isolated from col2.3 AR-transgenic mice. Calvaria were harvested from 8-10 week old mice and total RNA isolated. Gene expression was determined with qRT-PCR analysis for endogenous AR and for the colAR-transgene in both AR-transgenic lines. All data are expressed as mean ± SEM.

As expected, colAR transgene expression was elevated in bone (calvaria and tibia) with very low levels in all other tissues analyzed including muscle, skin, heart, kidney, fat, liver and tendon. We also determined AR-transgene expression in calvaria by qRT-PCR analysis using calvaria mRNA. Results show AR transgene expression 12 - 20 fold elevated relative to the endogenous AR gene for col3.6 and col2.3, respectively (Fig. 2). This result suggests similar levels of AR overexpression in both models.

AR-transgenic mouse phenotype:
   Comparison of body weight and length in col3.6 to col2.3 AR-transgenic mice: The effect of AR overexpression in distinct skeletal compartments on body weight gain was determined over a 6-month period in col3.6 (stromal lineage and osteoblast) and in col2.3 (osteocyte) AR-transgenic mice. The analysis reveals no difference in body weight in male col2.3 mice (Fig. 3). Interestingly, this result is in contrast to the reduced body weight phenotype we have observed in male col3.6 AR-transgenic mice, which we propose is the consequence of AR signaling in bone marrow stromal cells to alter lineage commitment and thus reduce adipogenesis (see Appendix 5).
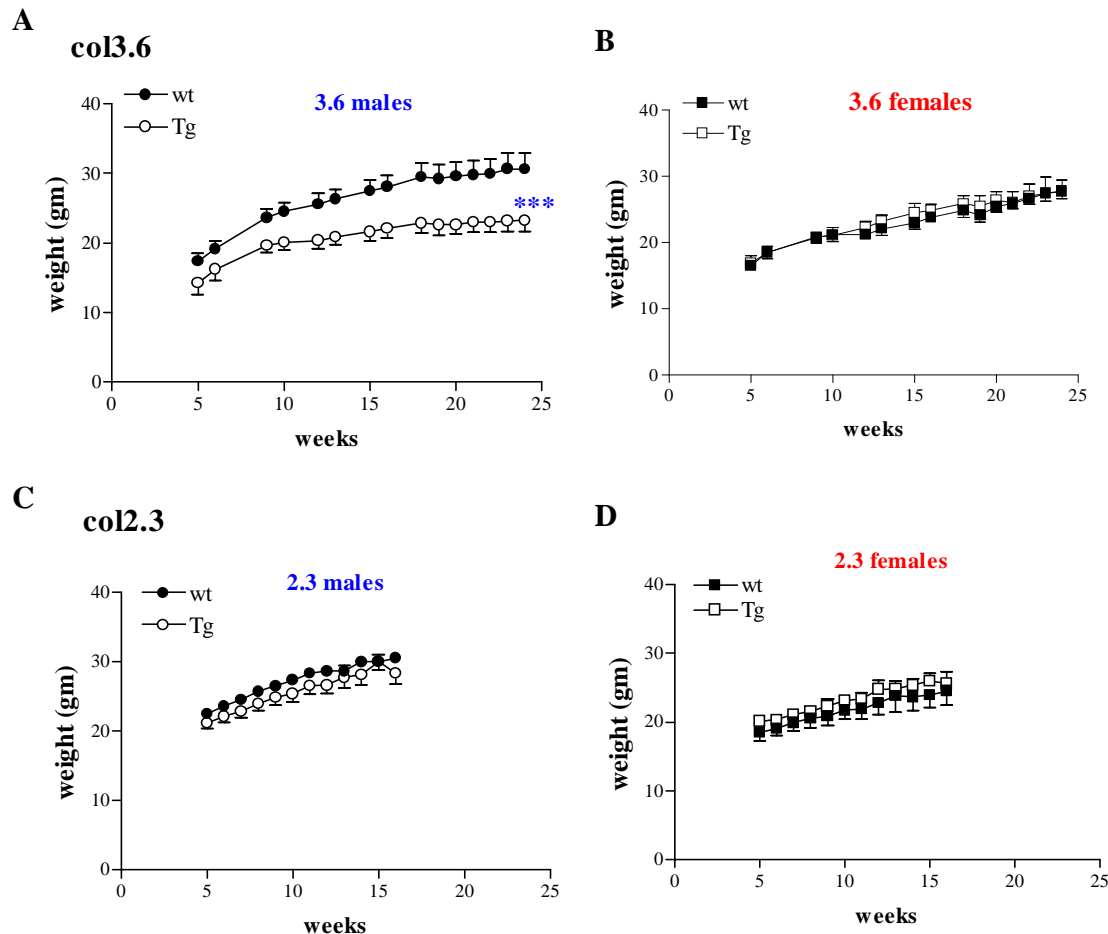

Fig. 3. Age-related changes in body weight in col3.6 vs. col2.3 AR-transgenic mice. Body weight determinations were carried out weekly over six months in both genders in both wild-type (wt) and AR-transgenic (tg) mice (n = 4-5). **A**. Weight gain in col3.6 AR-transgenic male mice. **B**. Weight gain in col3.6 female mice. **C**. Weight gain in col2.3 male mice. **D**. Weight gain in col2.3 female mice. All data are expressed as mean ± SEM.

Biochemical analysis: We next measured serum testosterone and 17ß-estradiol to determine whether AR-transgene expression influenced circulating hormone levels. Serum specimens from 2-month-old female and male mice of both genotypes were obtained under anesthesia by cardiac puncture (n = 9-10). Assays were performed by ELISA (Diagnostic Automation Inc). Levels of testosterone are higher in males than females, but there is no significant difference between littermate controls and AR-transgenic mice in either gender (data not shown). In addition, AR-transgene expression had no significant effect on estradiol levels. Serum calcium was determined using the complexone method (Raichem). There were no significant differences between col2.3 AR-transgenic and littermate controls in either gender. This lack of effect on circulating hormones is similar to observations in col3.6 AR-transgenic mice (10). However, as
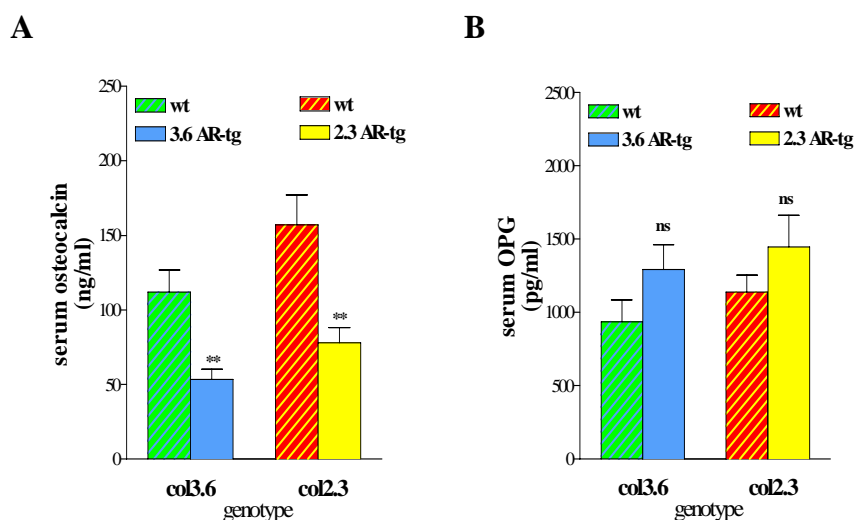
A   **B**

Fig. 4. Biochemical analyses of parameters of calcium metabolism and hormone levels in col2.3 vs. col3.6 AR-transgenic animals. Comparisons were performed between littermate control (wt) and AR-transgenic (tg) animals. Serum from 8-week-old mice (n = 9-10) was analyzed to determine levels of markers of calcium metabolism. **A**. Osteocalcin levels were significantly reduced in both male col2.3 and col3.6 AR-transgenic mice. **B.** OPG circulating levels were elevated in males but did not reach significance. Values are expressed as mean ± SEM. ******, $P < 0.01$; (*vs*. wild-type control).

shown in Fig. 4, there was a highly significant ~50% decrease in serum osteocalcin levels in male col2.3 AR-transgenic animals (*P*<0.001) quantitated by ELISA (Biomedical Technologies Inc) with no effect in females. Again this result is similar to that observed in col3.6 AR-transgenic mice (10). Since reductions in osteocalcin can reflect reduced bone turnover, these data are suggestive of a similar reduced turnover state in col2.3 mice as was observed in col3.6 AR-transgenic mice (10) but additional studies including dynamic histomorphometric analysis will be required to confirm this result. Finally, we also observed non-significant increase in serum osteoprotegerin (OPG) in col2.3 males as was previously described for col3.6 AR-transgenic males. Both col2.3 AR-transgenic families (219 and 223) showed similar elevations of OPG. Analysis of OPG and other osteoblastic or osteoclastic genes is currently being performed with total RNA harvested from both calvaria (intramembranous bone) and from long bone (tibia). This result is also consistent with preliminary in vitro analysis (see Appendix 4) demonstrating androgen treatment in osteocytic cells enhances OPG expression. Since OPG is a major regulator/inhibitor of osteoclastogenesis, the increase in OPG may indicate a reduction in osteoclast formation/activity, also consistent with reduced bone turnover. Further, this result is consistent with the hypothesis that mature osteocytes are primary mediators of androgen-mediated changes in OPG levels.

Histology staining for calvaria thickness: Calvaria were isolated from 2-month-old male and female col2.3 (osteocyte) AR-transgenic mice, and 5 µm sections were subjected to hematoxylin and eosin (H&E) staining after demineralization and paraffin embedding. Representative sections are shown. There was no observable difference in calvarial thickness between littermate controls and AR-transgenic mice in eit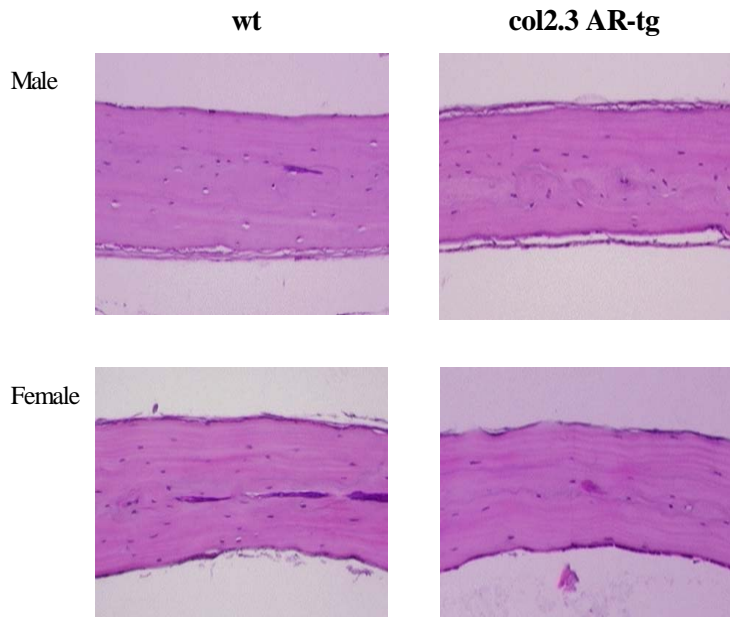her gender (Fig. 5), in contrast to an increase in calvarial thickness documented in male col3.6 (osteoblast lineage) AR-transgenic mice at the periosteal surface (10), as shown in Fig. 8A. Importantly, there is similar overexpression of AR levels in calvaria harvested from col3.6 vs. col2.3 mice (see Fig. 2). This result suggests that overexpression of AR in periosteal and immature osteoblasts is primarily responsible for increased bone formation in the calvaria. Dynamic histomorphometric and histological analyses are ongoing to characterize envelope-specific effects of col2.3 AR overexpression on periosteal vs. endosteal bone formation. We have previously determined an envelope-specific effect of col3.6 AR overexpression, with increased periosteal but dramatically inhibited endosteal apposition (10).

wt   **col2.3 AR-tg**

Male

Female

Fig. 5. Analysis of calvarial thickness in col2.3 AR-transgenic mice. Calvaria were harvested from 8-10 week old mice and processed for histological analysis by H&E staining. In contrast to col3.6 AR-transgenic mice, col2.3 do not demonstrate thickening at the periosteal surface.

Biomechanical analyses of col2.3 AR-transgenic mice: Femurs were isolated from 2-month-old male and female mice from col2.3 AR-transgenic mice. Whole femurs from both col2.3 AR-transgenic families (219 and 223) were broken in four-point bending to measure stiffness, maximum load, post-yield deflection (a measure of ductility/brittleness), and work-to-failure. Prior to testing, all femurs were also evaluated for morphology using microComputed Tomography (µCT). Contralateral femurs were fixed and embedded non-decalcified in poly-methylmethacrylate for analysis of osteoblastic and osteoclastic activity levels.

The biomechanical data shown in Fig. 6 clearly shows that AR overexpression throughout the osteoblast lineage (col3.6) has a more dramatic bone phenotype compared to AR overexpression in mature osteoblasts/osteocytes (col2.3). In all the analyses described below, there was no significant difference between *female* col2.3 AR-transgenic and littermate controls. The lack of effect in female col2.3 AR-transgenic mice is consistent with the higher level of endogenous testosterone in males vs. females that are presumably insufficient to result in significant transactivation of overexpressed AR. Thus, for the purpose of illustration, only data derived from male AR-transgenic mice are shown in Fig. 6. Male col2.3 AR-transgenic mice showed only an 8-9% reduction in stiffness and body-weight adjusted maximum load (whole bone strength) compared to controls, whereas the col3.6 AR-transgenic mice showed 33-36% reductions compared to controls. Both AR-transgenic lines showed a reduction in bone ductility (increased brittleness) assessed with post-yield deflection (48% for col3.6 and 31% for col2.3), and this in part leads to a reduction in work-to-failure (65% for col3.6 and 26% for col2.3). Thus,
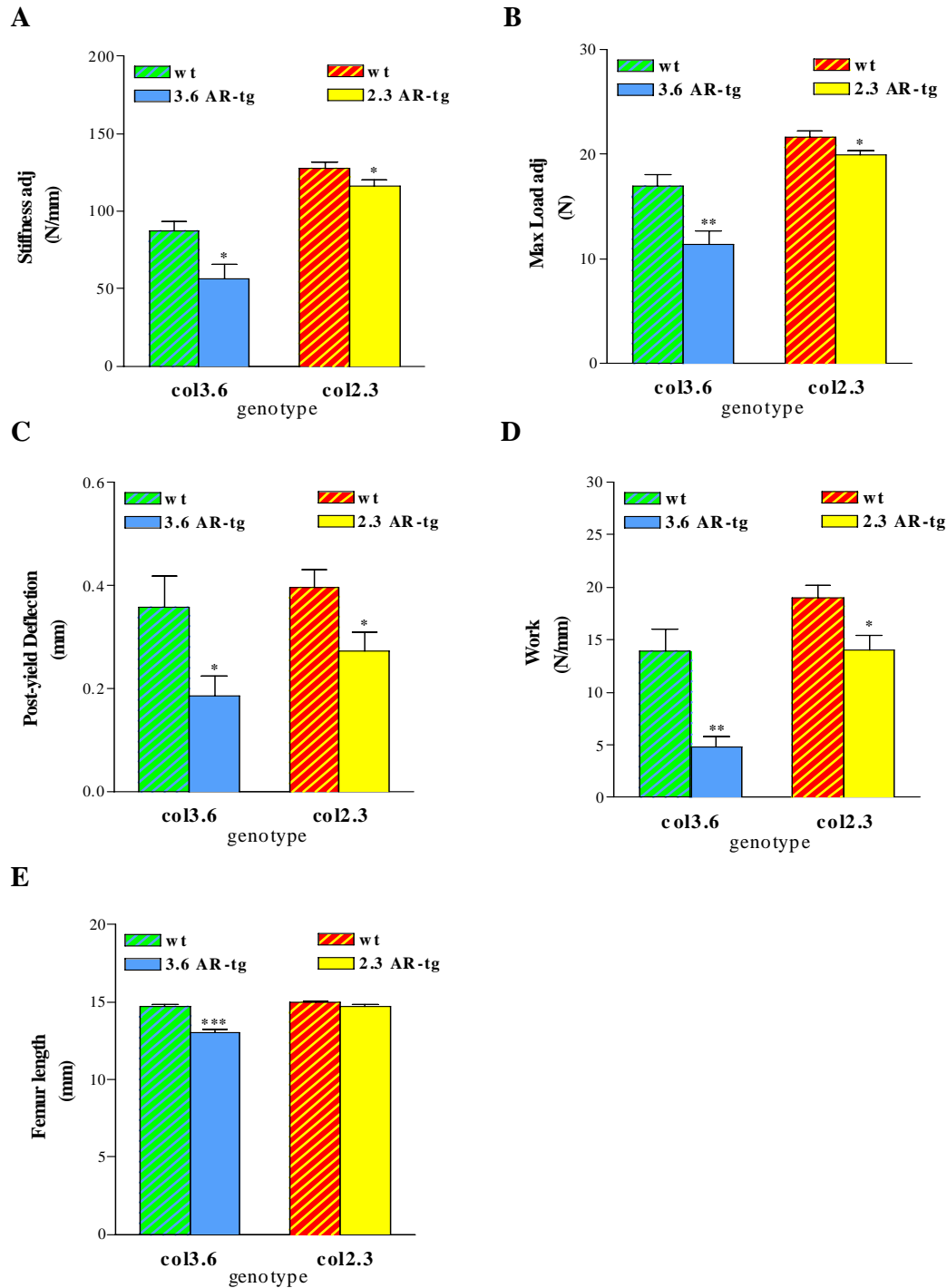
Fig. 6. Biomechanical analyses of bone quality in male col2.3 AR-transgenic mice. Femurs from wild-type (wt) and col2.3 AR-transgenic (tg) mice were isolated from 8-week-old mice to determine whole bone failure properties. Femurs were loaded to failure in 4-point bending and the stiffness, maximum load, and post-yield deflection were calculated from the load-deflection curves and adjusted for body weight differences. Data is presented as compared with published data characterizing the bone phenotype in col3.6 AR-transgenic mice. **A**. Adjusted Stiffness. **B**. Adjusted Maximum Load. **C**. Post-yield deflection. **D**. Work-to-failure. **E**. Femoral Length. The whole bone biomechanical properties are shown as mean ± SEM, n = 7-21. Differences between genotypes were determined by Student's two-tailed t-test using Welch's correction with Prism v4 software. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ (*vs.* appropriate wild-type controls).

the inhibitory effect on whole bone strength was more dramatic in col3.6 compared to the col2.3 male mice, but the reduction in ductility (increased brittleness) was fairly similar between the two models. These preliminary biomechanical data are consistent with the idea that increased AR signaling in mature osteoblasts (col2.3) may play a major role in mediating the effects of androgens on matrix quality and/or mineralization, but that immature osteoblast lineage cells (col3.6) are more important in determining overall whole bone strength. These changes in bone stiffness and strength will be confirmed when the µCT analysis is complete.

We have also begun histological analyses of distal femur from male and female col2.3 AR-transgenic and littermate controls (shown in Fig. 7, with males above and females below). These are stained sections where g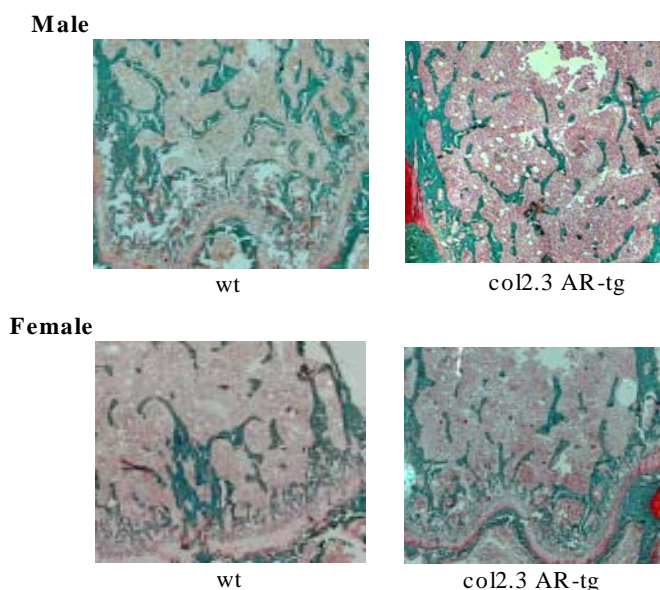reen = bone and red/pink = osteoid and soft tissue.   In comparison, the trabecular bone in the male col2.3 AR-transgenic and wild-type control appears similar, and clubbing/increased trabecular spicules as we have demonstrated in col3.6 AR-transgenic males (10) are not apparent at this level of analysis in col2.3 AR-transgenic mice. Females also appear basically the same. Ongoing analysis of studies proposed in Specific Aim 1 employ µCT and additional histological and static and dynamic histomorphometric analyses to fully characterize the trabecular and the cortical (both periosteal and endosteal) bone phenotype in col2.3 AR-transgenic mice in adulthood. Combined, these results are consistent with the development of a bone phenotype in male col2.3 AR-transgenic mice that is distinct from the phenotype in col3.6 AR-transgenic lines.

Male

wt   col2.3 AR-tg

Female

wt   col2.3 AR-tg

Fig. 7. Histological analysis of distal femur in col2.3 AR-transgenic (AR-tg) and littermate wild-type (wt) controls. Femurs were isolated from 8-10 week old mice, sectioned and stained for analysis. Unlike col3.6 AR-transgenic males, col2.3 AR-transgenic males do not appear to demonstrate increased trabecular bone formation or number in the distal metaphysis. As with col3.6 mice, there is little observable phenotype in females.

Androgen regulation of osteoblast apoptosis: We have also pursued studies to characterize the consequence of androgen signaling in mediating osteoblast apoptosis, as described in Specific Aim 3. We proposed the hypothesis that androgens positively influence the progression of osteogenesis to affect matrix quality, mediated by: a) AR transactivation in stromal cells, to provide key regulatory signals that regulate osteoblast but inhibit adipocyte determination, and b) differentiation-stage specific androgen action in osteoblasts, to regulate proliferation, matrix production, mineralization and apoptosis. In the studies summarized in the attached publication, we compared the effects of estrogen vs. androgen on osteoblast apoptosis. These studies demonstrate that estrogen treatment protects while androgen treatment exacerbates osteoblast apoptosis in both proliferating immature osteoblasts and in mature osteocytes. As shown in Fig. 8, in calvaria harvested from 2-month-old and even 6-month-old mice, male col3.6 AR-transgenic mice show enhanced TUNEL staining, indicative of
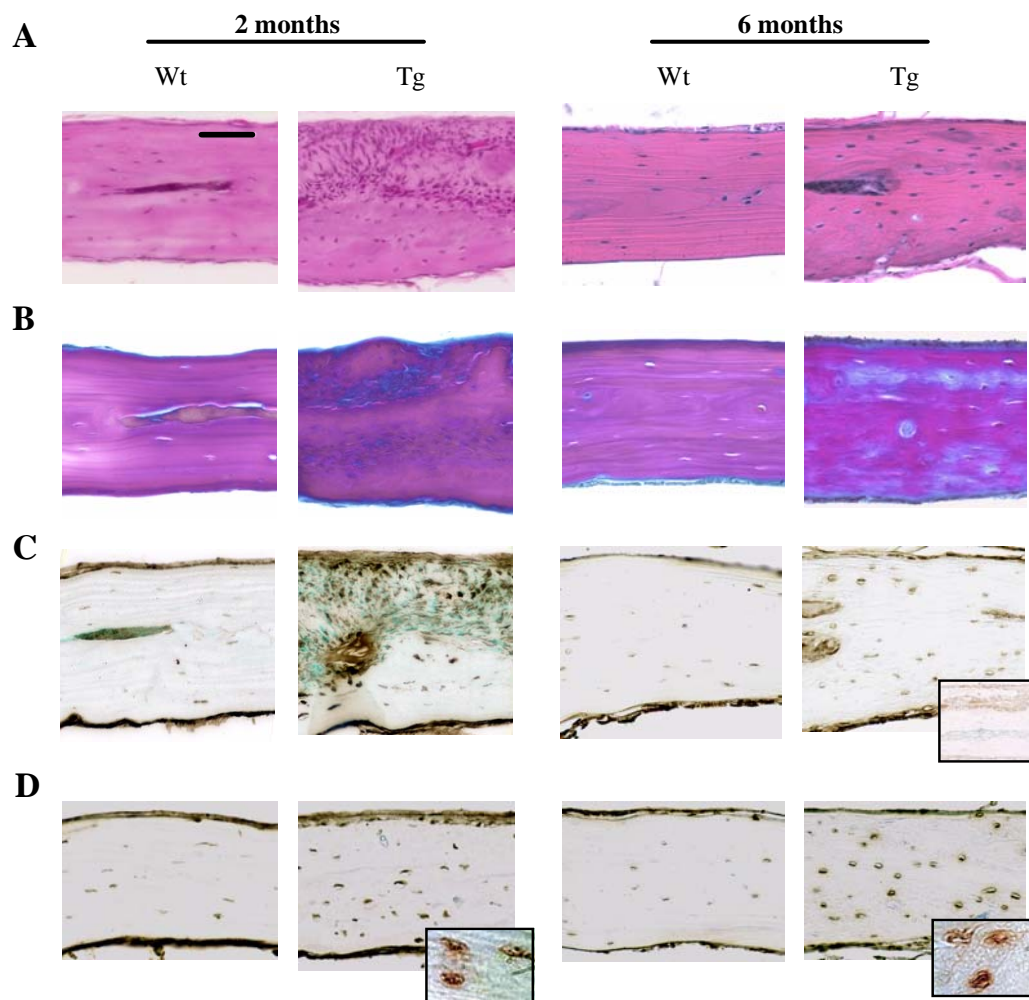
Fig. 8. Characterization of osteoblast apoptosis *in vivo* in col3.6 AR-transgenic mice. Calvaria from either 2 month old (*left panels*) or 6 month old (*right panels*) male col3.6 AR-transgenic or littermate control mice were fixed, decalcified, embedded and calvarial cross sections were evaluated. **A**. **Calvarial histology**. H & E stained cross sections show calvarial thickening in male AR-transgenic mice. Bar indicates 50µm. **B. New bone growth.** New bone growth associated with new collagen synthesis was identified in calvarial cross sections using van Geison staining. New collagen synthesis is shown in post-pubertal 2 month old mice and in adult 6 month old mice. **C**. **Analysis of apoptosis with TUNEL staining with new bone formation**. *In situ* end-labeling (TUNEL) staining was performed for analysis of apoptosis and counterstained with methyl green. Sections were derived from areas demonstrating new bone formation as described in C. Negative control sections for TUNEL analysis were incubated without TdT (*inset*). Quantitative analysis indicated ~10% apoptotic cells in wild-type and ~40% in AR-transgenic mice. **D**. **Analysis of apoptosis with TUNEL staining in osteocytic cells**. TUNEL staining was performed in calvarial outer regions that do not demonstrate thickening. Enhanced apoptosis was observed in male AR-transgenic mice both in areas with new bone formation and in matrix-embedded osteocytes. Higher power magnification shows osteoblasts morphology (*inset*). wt, wild-type mice (*left panels*); tg, AR-transgenic mice (*right panels*). Microscope magnification at 40X with higher power inset image at 100X.

enhanced apoptosis as a consequence of androgen signaling. This work has resulted in submission of both an abstract and a peer-reviewed publication (11). (Please refer to Appendices 2 and 3). As the mechanism of action of androgen on the skeleton remains controversial, we believe that this first report demonstrating discordant distinct effects of estrogen vs. androgen with respect to osteoblast apoptosis is interesting. Moreover, it provides further support for the concept that osteoblast apoptosis can be observed during new bone formation. These results are provocative, given the report demonstrating a critical role for caspase-3, an important apoptosis effector, in osteogenic differentiation (12). Our results are also supported by a recent publication of opposing effects of estrogen and androgen on apoptosis, here in T47D cells (13), and by gender-specific effects of Bcl-2 overexpression (14). Finally, these findings are of interest as they suggest a mechanism through which combination therapy with androgen plus estrogen treatment may be beneficial for skeletal health.

Finally, as a consequence of our studies on androgen action in osteoblasts and in particular the characterization of androgen signaling in the skeleton employing col3.6 AR-transgenic mice, we have recently published an invited review on the effects of androgen on bone formation after peer review (15) (please refer to Appendix 1).

Thus, the primary outcome of analyses through year one is that androgen action in the osteoblast lineage results in altered bone biomechanics and matrix quality, with envelope-specific effects on bone formation. Bone turnover appears to be reduced in both col2.3 and col3.6 AR-transgenic mice at 2 months of age, in the absence of changes in circulating steroids and without androgen administration. Distinct biomechanical properties are the consequence of androgen action that is restricted to mature osteoblasts/osteocytes, which suggests that much of the effect of androgen on bone matrix quality is mediated through signaling in mature osteocytes. In contrast, effects on bone formation in the periosteal compartment are likely mediated by immature osteoblast lineage cells. Compared with col3.6 AR overexpression throughout the osteoblast lineage (including bone marrow stromal cells, throughout osteoblast differentiation including osteocytes), col2.3 AR-transgenic males do not show reduction in femur length and demonstrate much less inhibition of whole bone strength properties including changes in stiffness, maximum load and work. However, col2.3 mice show similar changes indicative of low turnover, and similar increases in brittleness (decreased post-yield deflection), suggesting an analogous change in matrix quality and/or mineralization in both col2.3 and col3.6 AR-transgenic mice. Finally, androgen signaling in col3.6 mice exacerbates osteoblast apoptosis, even in areas of new bone formation. These results indicate that mature osteoblasts are important mediators of androgen action to influence matrix quality and influence turnover parameters, but immature osteoblast lineage and periosteal cells are the major contributors to envelope-specific alterations in bone formation. Thus, with increasing androgen action (as for example associated with anabolic steroid abuse) in young animals with still-growing skeletons, the bone matrix material is likely to be more stiff, less ductile and more damageable. Analyses in the mature adult will be necessary to extend these results into adulthood. Nevertheless, these results support the central hypothesis of distinct androgen signaling throughout osteoblast differentiation, and suggest that young anabolic steroid abusers may be at higher risk of stress fractures.

Key Research Accomplishments
Accomplishments for 2004-2005 directly characterizing androgen action in the skeleton:
    Peer reviewed publications:
    ▪   **Wiren KM**: Androgen action and bone growth: it's location, location, location. Curr Opin Pharmacol 5:626-632, 2005

- **Wiren KM**, Toombs AR, Semirale AA, Zhang X-W: Apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio. Bone *in press*, 2006

Abstracts:

- **Wiren KM**, Toombs A, Zhang X-W: Enhanced apoptosis associated with anabolic actions of androgens in AR-transgenic mice: requirement of reduced bcl-2/bax ratio. (Abstract #M226) J Bone Miner Res, 2004
- *****Wiren KM**, Toombs A, Kasparcova V, Harada S, Zhang X-W: Androgens inhibit osteoclast activity in AR-transgenic mice through reductions in RANKL/OPG ratio. (Abstract #F202/SA202) J Bone Miner Res, 2004
    - ***Selected for the Plenary Poster Session award**
- *****Wiren KM**, Toombs AS, Matsumoto AM, Zhang X-W: Androgen receptor transgenic mice have decreased body fat, reduced adipogenesis and an increased osteoblast differentiation program. (Abstract #1198) J Bone Miner Res, 2005
    - ***Selected for Oral Presentation**

Reportable Outcomes

Two peer-reviewed publications and three abstracts were published in 2004-2005 characterizing androgen action in the skeleton employing AR-transgenic mice. In the work presented here, we have also successfully created a second distinct AR transgenic family set with skeletally-targeted AR overexpression and have begun characterization of these mice at 2 months of age. In contrast to the original col3.6 AR-transgenic family, the col2.3 AR-transgenic family demonstrates overexpression that is limited to mature osteoblasts. Finally, the data generated by this grant provided evidence that AR signaling also influences developmental processes during skeletal modeling. This data helped secure an R01 grant from the NIH (DK067541) entitled, "Androgen action in bone: overexpression of AR". The goals of this R01 grant are to characterize the consequence of androgen signaling during early development.

Conclusions

These preliminary results have provided new insight into the importance of androgen action, through distinct AR transactivation, in mediating bone quality and changes that underlie envelope-specific responses. Our investigations of the mouse skeleton reveal that androgen signaling in immature osteoblasts and periosteal cells increases bone formation at the periosteal envelope (at least in calvaria) and influences processes that determine whole bone strength. In contrast, preliminary analysis presented here suggests that signaling in mature osteoblasts/osteocytes primarily mediates the effects of androgens on matrix quality and/or mineralization, and at least partially influences the effects of androgens to reduce bone turnover. These data suggest that a similar relationship may also exist in the human skeleton. Thus androgen action in the skeleton is complex and may not provide for improved skeletal dynamics in the still-growing skeleton, although the effect in mature adults and in the hypogonadal state is currently being evaluated. One of the side effects of enhanced androgen signaling is altered bone quality and susceptibility to damage in the young, which may be revealed under extreme physical conditioning such as that experienced during military training.

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Appendix 1 follows:

Wiren KM: Androgen action and bone growth: it's location, location, location. Curr Opin Pharmacol 5:626-632, 2005

ELSEVIER

Androgens and bone growth: it's location, location, location
Kristine M Wiren

Androgens increase bone mass in specific skeletal
compartments through effects on bone cells, enhancing
osteoblast activity but inhibiting that of osteoclasts. The
mechanism of action of androgens might involve both classic
androgen receptor transcriptional activation and rapid
non-genomic effects, and could also be dependent upon
low levels of estrogen.

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Introduction
Although it is well-established that androgens play a key
role in skeletal growth and turnover, the mechanisms
remain controversial given that the major androgen meta-
bolite, testosterone, is also a substrate for the production
of estradiol through aromatase activity. Additional com-
plications arise with the complex nature of bone itself.
There is, however, little controversy regarding anabolic
effects of androgens on one skeletal compartment: the
periosteal or outside surface of bone. Androgen action at
the onset of puberty is responsible for the development of
a sexually dimorphic skeleton (i.e. male bones are wider
than female) and is a significant determinant of bone
strength. Androgens are also important for the production
of peak bone mass in males. Cell types known to express
androgen receptors (ARs) include osteoblasts, osteoclasts
and mesenchymal stromal cells that differentiate toward
the osteoblast lineage. Proof-of-concept for AR action is
demonstrated in androgen-insensitivity models, with pro-
duction of a nonfunctional AR associated with a dramatic
reduction in total bone mass in humans even with excel-
lent estrogen compliance. AR null mice have high turn-
over osteopenia with increased formation but even
stronger enhancement of resorption. Targeted AR over-
expression in the skeleton further supports these findings,
as it results in enhanced periosteal formation, reduced

endosteal formation, increased trabecular bone formation
and a reduction of osteoclast activity, all without changes
in circulating steroid levels.

Although both estrogen and androgen circulate in men
and women, the influence of each on the remodeling
skeleton is distinct as shown by divergent responses to
gonadectomy in either gender and during modeling,
particularly with respect to bone size (periosteal apposi-
tion). Non-parallel pathways of action are also indicated
with the observation that combination therapy combining
estrogen and androgen is more beneficial than either
steroid alone in post-menopausal women [1–3]; this has
been confirmed in animal models [4•,5]. Estrogens are
thought to maintain adult bone mass predominantly
through inhibition of bone resorption by osteoclasts
(i.e. they act as anti-resorptive agents), which protect
the skeleton from further loss of bone. Non-aromatizable
androgens such as 5α-dihydrotestosterone (DHT), con-
versely, are anabolic agents that increase bone mass by
stimulation of bone formation, and thus represent an
important therapeutic class that has the potential to re-
build lost bone.

The purpose of this review is to discuss recently pub-
lished data regarding the anabolic effects of androgen on
bone growth, with a focus on the mechanisms by which
androgen regulates skeletal modeling and remodeling
through transactivation of the AR.

The complexity of bone: distinct compartments and cell types
The cell biology that underlies bone development is
complex. Two processes are evident in the development
of bone: endochondral formation from a cartilage anlage
as seen in long bones, and intramembraneous formation
seen in calvaria (skull cap). Long bones are organized in
compartments of cortical lamellar bone and trabecular
spiny or cancellous bone. Cortical bone is formed at mid-
shaft (diaphysis/cortical), whereas trabecular bone is
formed at the growing end (metaphysis/trabecular).
The cortical bone outer surface is the periosteal compart-
ment, and the inside surface is the endosteum. Periosteal
bone formation defines the cross-sectional area of bone,
whereas endosteal formation or resorption determines
cortical thickness. As described below and summarized
in Table 1, the effects of androgen on bone formation are
complex, and vary according to the specific skeletal site.

Androgen, the AR and bone cells
Ultimately, bone mass is determined by two processes:
formation and resorption. Distinct cell types mediate

Table 1

Androgen effects on bone formation.

Skeletal site	Response
Cortical bone	
Periosteal surface	Increase
Endosteal surface	Decrease
Trabecular bone	
Volume	Increase
Perimeter	Increase
Trabecular number	Increase
Trabecular spacing	Decrease
Trabecular thickness	Decrease/no effect
Intramembraneous bone	
Calvarial thickness	Increase

these processes. The bone-forming cell, the osteoblast, synthesizes bone matrix and regulates mineralization, and is responsive to most calciotropic hormones. The osteoclast is responsible for bone resorption. Although bone is clearly a target tissue with respect to androgen action, the mechanisms of action and cell types by which androgens exert their effects on bone biology are incompletely characterized. An additional complexity in terms of mechanism of action is that androgens can influence bone directly by activation of the AR, or indirectly after aromatization of androgens into estrogens with subsequent activation of estrogen receptors (ERs).

In vivo analysis has demonstrated significant expression of AR in osteoclasts and in all cells of the osteoblast lineage, including osteoblasts and osteocytes [6]. Data show preferential nuclear staining of ARs in males at sexual maturity, suggesting activation and translocation of the receptor in bone when androgenic steroid levels are elevated. Interestingly, ARs are also expressed in mesenchymal precursor cells [7•] — pluripotent cells that can differentiate into muscle, bone and fat. Androgen action can modulate precursor differentiation toward the osteoblast and myoblast lineage, while inhibiting differentiation toward the adipocyte lineage [8]. These effects on stromal differentiation might underlie some of the well-described consequences of androgen administration on body composition [9].

Evidence does suggest that androgens can act directly on the osteoblast. There are reports, some in clonal osteoblastic cell lines, of positive effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation [3]. Interestingly, the effect of non-aromatizable androgens on osteoblast proliferation is biphasic, with stimulation occurring initially but inhibition being seen with longer treatment times [10••]. This reduction in osteoblast viability, which can be inhibited with AR antagonists, might be associated with enhanced apoptosis, even in settings of enhanced bone formation (K Wiren, unpublished). Androgens may also augment the

osteoanabolic effects of mechanical strain in osteoblasts (see also Update) [11].

Analysis of AR, ERα and ERß mRNA and protein expression during osteoblast differentiation *in vitro* show that all receptors display expression patterns dependent upon the stage of differentiation [12]. The levels of AR expression increase throughout osteoblast differentiation, with the highest AR levels seen in mature osteoblast/osteocytic cultures, suggesting that androgen may predominantly affect mature osteoblasts. Thus osteoblast differentiation and steroid receptor regulation are intimately associated. Androgen exposure has frequently been shown to upregulate AR expression in osteoblasts [13], suggesting increased AR levels and responsiveness as androgen concentrations increase.

Potential modulation of osteoclast action by androgens is suggested by reports of AR expression in the osteoclast [6]. In addition, androgen treatment reduces bone resorption of isolated osteoclasts, inhibits osteoclast formation [14], including that stimulated by parathyroid hormone [15], and might play a direct role in regulating aspects of osteoclast activity in both AR null mice [16] and AR transgenic mice [17••]. Indirect effects of androgen to modulate osteoclasts are indicated by the increase in osteoprotegerin by testosterone treatment in osteoblasts (see also Update) [18]. Androgen levels are likely to be a less significant determinant of bone resorption *in vivo* than those of estrogen [19,20], although this remains controversial [21].

There has been speculation that the positive effects of androgens on the skeleton may be mediated through non-specific, non-genomic actions [22], including non-genomic AR signaling in osteoblasts [23]. Recent data, however, suggest that genomic signaling may be the more significant regulator in bone [24–26].

The effects of sex steroids in the development of a sexually dimorphic skeleton
During childhood and adolescence, skeletal development is characterized by marked expansion of cortical proportions and increasing trabecular density. During this process, the skeleton develops distinctly in males and females, particularly at the periosteal surface. Sex differences in skeletal morphology and physiology occur at or around puberty, with little effect of gonadal steroids prior to puberty [27]. For that reason, it is hypothesized that gender differences, particularly with respect to 'bone quality' and architecture (i.e. predominantly bone width) are modulated by the sex steroids estrogen and androgen. Thus, a distinct response to estrogen and androgen has been described *in vivo* especially in cortical bone. At the periosteum, estrogen suppresses, whereas androgen stimulates, new bone formation yet, conversely, at the endosteum estrogen stimulates, but androgen strongly

suppresses, formation [17[••]]. Interestingly, low levels of estrogen (in the obligate presence of androgen) might also be important for stimulation of periosteal bone formation during development [28[•]]. Young men do have larger bone area than women, particularly at peripheral sites [29]. Thus, estrogen decreases, but androgen increases, radial growth in cortical bone (see also Update). These distinct responses to estrogen and androgen probably play an important role in determining sexual dimorphism of the skeleton (i.e. male bones being wider but not thicker than those of females) [30]. Androgens are also essential for the production of peak total-body bone mass in males [31[•]].

The importance of androgens in bone formation: human studies

It is also clear that androgens play an important role in maintaining the adult skeleton. Hypogonadism in both sexes is associated with bone loss. Replacement therapy with estrogen, aromatizable testosterone or non-aromatizable androgen (e.g. DHT) are all generally effective at ameliorating this loss. Clinical data demonstrate that estrogen replacement therapy tends to suppress bone resorption with only modest effects on bone formation. By contrast, androgen replacement data have demonstrated clear effects on bone formation. The skeletal response to androgen administration has been characterized as increased cortical and trabecular bone mass, together with increased bone width with surface periosteal expansion but a lack of inner endosteal deposition, in the setting of inhibition of resorption owing to reduced osteoclast activity [19,21,32,33[••]]. Thus, androgens act to maintain trabecular bone (see also Update) and expand cortical bone. In female-to-male (genetic female) transsexuals, high-dose testosterone therapy resulted in increased bone mineral density (BMD) at the femoral neck, with estradiol declining to post-menopausal levels [34]. In a second study, the BMD of female-to-male transsexuals treated with 'male' levels of testosterone increased to normal male levels at cortical sites [35]. Finally, men with constitutional delay of puberty have impaired periosteal expansion [36[•]].

The global contribution of ARs to bone quality in humans has been assessed in women with androgen insensitivity syndrome (AIS) with 46,XY genotypes. In such patients, AR abnormalities produce an impaired (to complete) lack of response to endogenous and exogenous androgens. Women with AIS who were rated to have been poorly compliant with estrogen therapy showed significantly lower bone mass (indicated by BMD) than those with good or excellent compliance ratings [37]. However, even those women who 'promptly started and fastidiously maintained estrogen replacement' showed a significant deficit both in lumbar spine BMD and in lumbar and femoral bone mineral apparent density, which largely corrects for the confounding effects of bone size. Thus,

the lack of direct skeletal actions by androgen underlies at least part of the bone mass deficits observed in women with complete AIS. Although aromatization of testosterone to estrogen metabolites can play an important role in mediating the effects of androgens in bone, the significance of AR action independent of ER in mediating androgen effects is clear [38].

Animal studies: androgen administration and AR null and transgenic mice

Results from animal studies also support an effect of androgen on bone formation. Experimental strategies, such as surgical or pharmacological intervention, and examination of genetic models have all been employed to characterize androgen signaling. Distinct effects of androgen are seen with gonadectomy when comparing the effects of orchidectomy (ORX) in male versus ovariectomy (OVX) in female rats. OVX and the associated loss of sex steroids in the female generally results in decreased trabecular area with increased osteoclast number. In cortical bone, an increase in bone formation at the periosteal surface is seen with circumferential enlargement, but a decrease is seen in endosteal bone formation. These results demonstrate that estrogen protects trabecular bone predominantly through inhibition of osteoclast activity/recruitment, but has an inhibitory action at the periosteal surface (for example, see [39[••]]). In male rats, ORX with the attendant loss of sex steroids also results in decreased trabecular area with increased osteoclast number. However, in contrast with females, periosteal formation in cortical bone is reduced with the loss of androgen. Androgen treatment is effective in suppressing the acceleration of bone remodeling normally seen after ORX [40]. Histomorphometric analysis of androgen action in ORX male mice has shown that the bone-sparing effect of AR activation in trabecular bone is distinct from that of ERα at the same site. Thus, AR activation preserves the number of trabeculae but does not preserve thickness or volumetric density, or mechanical strength in cortical bone [41]. In the intact animal, stimulation of endosteal formation by estrogen compensates for the lack of periosteal formation, resulting in no difference in biomechanical strength between the sexes. Nevertheless, factors that influence periosteal apposition might constitute an important therapeutic class, as periosteal bone formation is often a neglected determinant of bone strength [42]. In addition, it appears that ORX affects cranial development more than OVX [43], suggesting that androgen action is particularly important in intramembranous bone.

The specific contribution of AR signaling *in vivo* has been approached in genetic animal models with global AR modulation, including the testicular feminization (Tfm) model of AIS [39,44] and with non-targeted AR knockout mice [16,45]. In the Tfm model, ORX demonstrates the importance of AR in mediating the positive effects of androgen in maintenance of trabecular bone, and of

cortical bone particularly at the periosteal surface [39,44]. The bone phenotype that develops in a global AR null male mouse model is high-turnover osteopenia, with reduced trabecular bone volume and a significant stimulatory effect on osteoclast function [16,45,46]. As expected, bone loss with ORX in male AR null mice was only partially prevented by treatment with aromatizable testosterone owing to the lack of AR.

A final model for AR modulation is represented by modest overexpression of AR in AR transgenic mice, constructed with full-length AR under the control of the 3.6 Kb type I collagen promoter [17••]. AR transgenic mice are the only model with skeletally targeted manipulation of AR expression, and demonstrate enhanced sensitivity to androgen without changes in circulating steroids or androgen administration. Bone-targeted AR overexpression results in a complex phenotype, predominantly in males, with increased trabecular bone mass (increased trabecular number but not thickness) in the setting of inhibition of resorption caused by reduced osteoclast activity. In addition, cortical formation is altered, with surface periosteal expansion but a lack of inner endosteal deposition. Inhibition of osteoclastic resorption might be responsible for altered trabecular morphology, consistent with reduced osteoclast activity and increased trabecular bone volume observed with androgen therapy in humans. The dramatic inhibition at the endosteal envelope could be responsible for the modest decrease in cortical bone area and changes in biomechanical properties observed. Thus, the bone phenotype observed in AR transgenic mice is consistent with many of the known effects of androgen treatment on the skeleton (Table 1). These results indicate that AR expressed in bone can be a direct mediator of androgen action to influence skeletal development and homeostasis.

Potential therapeutic options for bone

Testosterone treatment has been shown to be effective in stimulating bone turnover in boys with growth or pubertal delay [31•,47], and at ameliorating bone loss during aging in men with low testosterone levels [48•,49]. Nevertheless, androgen replacement in hypogonadal men remains a controversial issue [50] even though low testosterone is associated with increased mortality risk in geriatric patients [51]. It appears that androgens in a form that cannot be 5α-reduced might be preferable to satisfy concerns over prostate hypertrophy [40], as it appears that treatment with testosterone can be as effective as DHT with respect to preservation of bone mass [52•,53]. Testosterone may be effective at reducing bone formation in women with anorexia nervosa [54]. Furthermore, administration of androgenic anabolic steroids to stimulate bone mass shows promise in the treatment of severely burned children that normally develop growth inhibition or arrest [55•], and possibly in wasting diseases associated with androgen deficiency and reduced bone mass, such as HIV [56]. Anabolic steroids, however, might not be

effective in all settings [57]. Finally, selective androgen receptor modulators, analogous to selective estrogen receptor modulators used to influence estrogen receptor signaling, are being developed to protect bone and muscle but not stimulate prostate growth [58]. Although no selective androgen receptor modulators have been clinically approved, data indicate a bone protective effect in animals [59,60] and they may thus provide a new therapeutic option in the near future.

Conclusions

Androgens are important in the maintenance of a healthy skeleton, and have been shown to stimulate bone formation (Table 1). Androgens influence skeletal modeling and remolding by multiple mechanisms through effects on osteoblasts and osteoclasts and an influence on the differentiation of pluripotent stem cells toward the osteoblast lineage. The specific effects of androgen on bone cells are mediated directly through an AR signaling pathway, but there are also indirect contributions to overall skeletal health through aromatization and ER signaling. Androgens therefore provide promise for treatment in settings of low bone mass.

Update

As noted, androgens increase muscle mass and have thus been postulated to influence bone density through skeletal loading. Although it has been shown that appendicular skeletal muscle mass is positively correlated with bone mineral content and areal BMD in men [61•], controversy remains regarding the influence of fat mass versus muscle mass on bone formation in both sexes during aging and at specific sites [62•].

Interestingly, recent data demonstrate both direct inhibition of osteoclast formation and resorption by androgen treatment and an indirect effect through increased formation of osteoprotegerin via osteoblasts. In contrast, estrogen treatment had no direct effect on osteoclasts, whereas a similar indirect stimulation of osteoprotegerin was noted in osteoblasts [63••].

Additional support for the notion that androgens increase, whereas estrogens reduce, cortical bone size through opposing effects at the periosteal surface is reported in a recent cross-sectional study of 1068 young men [64•]. Notably, the apparent free testosterone concentration was found to be a positive predictor of cortical cross-sectional area and periosteal circumference, whereas free estradiol was an independent negative predictor of cortical bone size.

Using magnetic resonance microimaging to characterize trabecular architecture *in vivo*, Benito *et al.* [65••] show in a small sample of hypogonadal men that testosterone replacement results in improved trabecular connectivity, including an increase in trabecular thickness.

Acknowledgements

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Appendix 2 follows:

Wiren KM, Toombs AR, Semirale AA, Zhang X-W: Apoptosis associated with androgen action
in bone: requirement of increased Bax/Bcl-2 ratio. Bone *in press*, 2006

Osteoblast and osteocyte apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio*

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Abbreviated title: androgen stimulates osteoblast apoptosis

ABSTRACT: Both the number and the activity of osteoblasts are critical for normal bone growth and maintenance. Although a potential role for estrogen in protection of bone mass through inhibition of osteoblast apoptosis has been proposed, a function for androgen is much less clear. The aim of this study was to establish a direct role for androgen to influence osteoblast apoptosis both *in vitro* and *in vivo*. AR-MC3T3-E1 cells, with androgen receptor (AR) overexpression controlled by the type I collagen promoter, were treated with the non-aromatizable androgen 5α-dihydrotestosterone (DHT). Apoptosis was assessed by three different techniques including DNA fragmentation, caspase-3 activation and changes in mitochondrial membrane potential. Transactivation of AR by DHT enhanced apoptosis while 17ß-estradiol (E_2) treatment reduced apoptosis in both proliferating preosteoblasts and mature osteocyte-like cells. To explore mechanism, the apoptosis regulators Bcl-2 (antiapoptotic) and Bax (proapoptotic) were evaluated. Western analysis revealed that DHT decreased Bcl-2 resulting in a significantly increased Bax/Bcl-2 ratio. Regulation of Bcl-2 was post-transcriptional since *bcl-2* mRNA levels were unaffected by DHT treatment. Furthermore, ubiquitination of Bcl-2 was increased and serine phosphorylation was reduced, consistent with inhibition of MAP kinase signaling by DHT. Increased Bax/Bcl-2 ratio was essential since either Bcl-2 overexpression or Bax down-regulation by RNA interference (RNAi) partially abrogated or reversed DHT-enhanced osteoblastic apoptosis. In order to establish physiologic significance *in vivo,* AR-transgenic mice with AR overexpression in the osteoblast lineage and thus enhanced androgen sensitivity were characterized. In male AR-transgenic mice, increased osteoblast apoptosis was observed *in vivo* even in association with new bone formation. Thus, although estrogen can be anti-apoptotic, androgen stimulates osteoblast and osteocyte apoptosis through an increased Bax/Bcl-2 ratio even in anabolic settings. These results identify a new mechanism for androgen regulation of osteoblast activity distinct from estrogen, and suggest that enhanced apoptosis can be associated with anabolic stimulation of new bone growth. Androgens thus play a distinct role in skeletal homeostasis.

Keywords: osteoblast, apoptosis, androgen receptor, androgen, estrogen

INTRODUCTION

The skeleton is an important target organ for androgen action, distinct from estrogenic effects.

Androgen receptors (AR) are expressed in osteoblasts, the cells responsible for osteogenesis. AR

levels are increased in osteoblasts after androgen exposure (56, 58) and as cells differentiate into a

mature osteocytic phenotype (55). The importance of AR signaling to influence skeletal

homeostasis has been clearly demonstrated in both human (32) and mouse (51) models with

androgen insensitivity, and in global AR null models (42, 62). Characterization of AR-transgenic

mice with skeletally targeted AR overexpression has demonstrated proof of principle that direct

androgen signaling in the osteoblast is an important mediator of androgen action in bone (59).

While the association of androgen with skeletal health and normal bone homeostasis is well

established (for review, see 52), the physiologic responses and molecular pathways influenced by

androgen in bone remain poorly characterized. The influence of estrogen on bone cell life span is

an area of active investigation since it may be important in maintenance of bone mass, and play a

role in the development of osteoporosis (48). However, the effect of androgen is not well

understood and is complicated by the fact that testosterone is the substrate for estradiol synthesis

through aromatase activity. Thus, a controversy exists surrounding androgen's specific role(s) in

bone homeostasis.

Androgen has been shown to influence bone cells in a complex fashion. For example, the effect

of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement

following short or transient treatment but significant inhibition following longer treatment (see 57).

Continuous treatment with the non-aromatizable androgen 5α-dihydrotestosterone (DHT) in

proliferating preosteoblasts also resulted in decreased cell viability (57). Reduced viability was

associated with overall reduction in mitogen-activated (MAP) kinase signaling and with inhibition of

elk-1 gene expression, protein abundance and extent of phosphorylation. Importantly, inhibition of

MAP kinase signaling was observed both in normal primary rat calvarial cultures and in a model of

enhanced androgen responsiveness, AR-MC3T3 cells. This result contrasts with stimulation of MAP

kinase signaling and AP-1 transactivation observed with brief androgen exposure, that may be mediated through non-genomic mechanisms (24, 28, 63). Since enhanced apoptosis is frequently associated with inhibition of MAP kinase signaling, the aim of the present study was to define the effects of androgen administration on osteoblast apoptosis *in vitro* and *in vivo*.

Apoptosis, or programmed cell death, is an essential process that is important for maintaining tissue homeostasis under normal conditions, in response to environmental insults and plays a vital role during embryogenesis that includes shaping of developing tissues (1). In bone, apoptosis is important during embryonic limb development, skeletal maturation and modeling, adult bone turnover by remodeling, and during fracturing healing and regeneration (19). One of the best characterized mechanisms to induce cell death is the release of proapoptotic proteins from the mitochondria into the cytosol (for a recent review, see 4). Members of the Bcl-2 family, including Bcl-2 and Bax, are central regulators of apoptosis that promote (Bax) or inhibit (Bcl-2) cell death. Although a competition between Bax and Bcl-2 exists, each is able to regulate apoptosis independently (26); thus the ratio of Bax/Bcl-2 is an important indicator of apoptosis.

Previous reports indicate that Bcl-2 can be phosphorylated, and that phosphorylation of Bcl-2 is closely associated with regulation of apoptosis (8, 14). For example, positive regulation of Bcl-2 levels through MAP-kinase mediated phosphorylation has been demonstrated, with activation of p44/42 MAP kinase inhibiting both downregulation of Bcl-2 protein and subsequent apoptosis (41). The converse has also been observed, with inhibition of MAP kinase signaling shown to reduce Bcl-2 phosphorylation at consensus MAP kinase sites, leading to enhanced ubiquitination and ensuing Bcl-2 degradation in proteasomes (5, 9). Androgen treatment has been reported to reduce Bcl-2 levels (6, 20) and enhance apoptosis (54, 64) in some tissues. Although we have demonstrated androgen-mediated reductions in MAP kinase signaling with chronic treatment (57), the impact of androgen treatment on levels of antiapoptotic Bcl-2 in osteoblasts has not been previously characterized.

Most analyses regarding effects of sex steroids on osteoblast apoptosis indicate that estrogen may be important in inhibiting apoptosis to increase osteoblast life span (39). Few studies have directly analyzed the specific effects of androgens however, leading to controversy regarding the nature of androgen signaling. In addition, it has been postulated that AR and estrogen receptor (ER) can activate an antiapoptotic non-genomic signaling pathway in osteoblasts with the same effectiveness regardless of whether the actual steroid ligand is an androgen or an estrogen (27). Recent data however suggests that distinct, genomic signaling may be the more significant regulator *in vivo* (36, 44, 50). In this study, the specific effect of androgen on osteoblast apoptosis through AR transactivation was examined *in vitro* with AR-MC3T3 cultures and *in vivo* in AR-transgenic mice. Interestingly, one facet of the low turnover bone phenotype observed in young male AR-transgenic mice is an anabolic response to androgen signaling (59), seen with an enhanced bone formation rate at the periosteum of long bones and with calvarial thickening. The effect of androgen on osteoblast apoptosis in an anabolic setting has not previously been examined. To gain further insights into the specific effect of androgen vs. estrogen on osteoblast apoptosis, we employed two novel models of enhanced androgen responsiveness; clonal col3.6 AR-MC3T3 cells (hereafter referred to as AR-MC3T3) stably transfected with full-length AR under control of 3.6 kb of the type I collagen promoter, and male col3.6 AR-transgenic mice (hereafter referred to as AR-transgenic mice). Results indicate dichotomous regulation of osteoblast apoptosis by androgen vs. estrogen, with chronic administration of androgen stimulating but estrogen inhibiting apoptosis.

MATERIALS AND METHODS:

*Reagent*s

   All the media, buffers, supplements and reagents for cell culture were obtained from GIBCO BRL-Life Technologies (Grand Island, NY) or Sigma Chemical Co. (St. Louis, MO). Steroid hormones and other reagents were obtained from Sigma Chemical Co. The active metabolite hydroxyflutamide (α, α, α-trifluoro-2-methyl-49-nitro-*m*-lactotoluidide, SCH 16423) was kindly provided by Schering-Plough Corp. (Madison, NJ). Hydroxyflutamide, an androgen receptor antagonist, was added to the cultures 30 min before hormone addition.

Cell culture

   Cells stably transfected with AR (AR-MC3T3) or ß-galactosidase (ßgal-MC3T3) under the control of the rat 3.6 kb α1(I)-collagen promoter were created as previously described (57). Cultures were maintained in minimal essential media (MEM) with 2.38 g/L HEPES and 2.2 g/L NaHCO$_3$ buffer, supplemented with 5% calf serum (CS). These cells were maintained throughout the study in the presence of G418 geneticin sulfate at 500 µg/ml. Cultures were treated with steroids in 5% charcoal-stripped CS. For appropriate osteoblast differentiation, cultures were switched at confluence to media containing 50 µg/ml ascorbic acid and 10 mM ß glycerophosphate. Osteocytic cultures were derived from cells grown 20-30 days (see 31, 55, 61). Steroids were dissolved as stocks in ethanol and used at concentrations from 10^{-12} M to 10^{-8} M. The final ethanol concentration in the media was no higher than 0.1%.

Transient transfection, luciferase reporter assay

   The potential of E$_2$ to stimulate ER transcriptional activation was determined using the E$_2$ response element (ERE) basal promoter-luciferase (4ERE-TATA-luciferase; ERE-luc) reporter construct containing 4 EREs linked to a minimal promoter, kindly provided by Dr. David Shapiro. ERE-luc was used to confirm that overexpression of AR did not influence transactivation of other

steroid hormone receptors. AR-MC3T3 cells were grown in 6-well plates for 24 h before transfection. AR-MC3T3 cells were transfected with FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) using 1.5 µg of ERE-luc and 0.5 µg ßgal expression vector for normalization in each well. Cultures were treated with vehicle or 10^{-12} -10^{-8} M E_2, with combined treatment with E_2 and 10^{-8} M DHT, or with DHT alone for 54 h. Cells were harvested and extracted in 300 µl reporter lysis buffer (Promega Corp, Madison WI). Luciferase and ßgal activities were analyzed as previously reported (57). The data shown represent the mean $\pm$ SEM from triplicate samples, performed in independent transfections two to three times.

Western blot analysis and immunoprecipitation

Protein was extracted from proliferating cell cultures as previously reported (55). Polyclonal rabbit antibody recognizing AR (PA1-111A, Affinity Bioreagents Inc.) was used at 4 µg/ml. Both Bcl-2 and anti-mouse Bax polyclonal antibody (Δ21) specific for all 1-171 amino acids but the carboxy-terminal 21 amino acids (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were diluted at 1:200. As a loading control for total protein, Western blot analysis was performed with an anti-α tubulin antibody at 1:1000 (Sigma, Saint Louis, MO). Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Bio-Rad Laboratories, Richmond, CA) at 1:2000. Bound antibodies were visualized by ECL (Amersham Pharmacia Biotech, Piscataway, NJ) on Kodak X-AR5 autoradiographic film.

For the detection of phosphorylated or ubiquitinated forms of Bcl-2, AR-MC3T3 cells were treated with 10^{-8} M of DHT for 72 h. To stimulate extracellular signal regulated kinase (ERK) activity, cultures were first starved in serum-free medium for 1 h with a subsequent addition of 5% charcoal stripped serum for 2 h. Equal amounts of protein were immunoprecipitated with anti-Bcl-2 antibody. Immunocomplexes were collected with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.), resolved by 12% SDS-PAGE, and transferred onto Immobilon-P PVDF membrane. Western blot analysis after immunoprecipitation was carried out with either anti-phosphoserine-specific antibody

at 0.1 μg/ml (clone 4A9; Alexis Biochemicals, San Diego, CA), anti-Bcl-2 antibody as described above, or anti-ubiquitin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:300 dilution. Western blot analysis of extracts from AR-MC3T3 cells 48 h following mock transfection or transfection with the Bcl-2 expression plasmid or Bax siRNA were also performed. Quantitative analysis of the proteins was performed by volume densitometry using Optiquant Software (PerkinElmer Life and Analytical Sciences, Inc, Boston, MA) after scanning of the film (ScanMaker 9800XL, Microtek, Carson, CA) in the linear range. Apoptosis data was expressed as the Bax/Bcl-2 ratio. Immunoprecipitation data is presented as the protein to α-tubulin ratio to correct for variations in protein loading, and then normalized to control values for comparison between treatments. The data shown represent the mean ± SEM.

AR quantification

Both AR-MC3T3 and control ßgal-MC3T3 stable cultures were grown for 5 to 25 days, and AR binding analysis was performed as previously described (57). ARs were quantitated by titration analysis using [^{3}H]methyltrienolone ([^{3}H]R1881) (New England Nuclear, specific activity 70-87 Ci/mM). Specific [^{3}H]R1881 binding capacity was estimated by one site binding with non-linear regression and Scatchard plot analysis using Prism v4 software (GraphPad Software, Inc, San Diego, CA). The high affinity (K_d = 1.0-5.0 x 10^{-10} M), specific binding of [^{3}H]R1881 was expressed in fmol/mg DNA and sites/cell. The sensitivity for the detection of specific androgen binding using these methods was 5 fmol/ml.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and primer sequence

Confirmation of endogenous AR and colAR transcript expression performed using qRT-PCR with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) on DNase-treated total RNA

as previously described (59). AR transgene mRNA was amplified with 5'-GCATGAGCCGAAGCTAAC-3' and 5'-GAACGCTCCTCGATAGGTCTTG-3'. These primers were designed to specifically amplify the transgene using sites in the collagen untranslated region and part of the AR sequence. Endogenous mouse AR mRNA was amplified with 5'-GGAATTCGGTGGAAGCTACA-3' and 5'-CCGGGAGGTGCTATGT-3'. To determine the mRNA levels of *bcl-2* and *bax* after treatment with DHT or E_2, murine *bcl-2* mRNA was amplified with 5'-TTCGCAGAGATGTCCAGTCA-3' and 5'-CACCCCATCCCTGAAGAGTT-3'; murine *bax* mRNA was amplified with 5'-CCAAGAAGCTGAGCGAGTGT-3' and 5'-TGTCCACGTCAGCAATCATC-3'. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method after normalizing expression with either 18S rRNA or fluorescence to the specific RNA binding dye RiboGreen (Molecular Probes, Eugene, OR, USA) as previously described (15). Real-time qRT-PCR efficiency was determined for each primer set using a five-fold dilution series of total RNA and did not differ significantly from 100%. Following PCR, specificity of the PCR reaction was confirmed with melt curve analysis. Amplicons were also sequenced for confirmation. Data is presented as mean $\pm$ SEM.

Assessments of apoptosis

AR-MC3T3 cells were plated in 6-well dishes and grown for either 5 days (proliferating) or 29 days (osteocyte-like) in the continuous presence of either vehicle or 10^{-8} M DHT or 10^{-8} M E_2. Apoptosis was induced either with 50 µM etoposide (ETOP) for 18 h before harvest or by serum starvation for 48 h, replacing serum with 0.1% bovine serum albumin. Three independent methods were employed for analysis of apoptosis after these treatments.

Cytoplasmic nucleosome enrichment: Quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in combined attached and detached cell pools of AR-MC3T3 treated as described above used the Cell death ELISA[Plus] (Roche

Diagnostics, Indianapolis, IN) kit. Absorbance was measured at 405nm, and data are expressed as the nucleosome enrichment factor = A $_{treated}$/A $_{untreated}$.

Caspase-3/CPP-32 activity: Apoptosis-induced activation of caspase-3 activity in treated AR-MC3T3 cultures was assessed by measuring colorimetric substrate DEVD-*p*NA cleavage (BioVision, Inc.) according to manufacturer's suggestions in a homogenate containing 50 µg protein.

Mitochondrial membrane potential: Loss in mitochondrial membrane potential ($\Delta\Psi_{mito}$) in treated AR-MC3T3 cells after induction of apoptosis was detected using ApopAlert Mitochondrial Membrane Sensor kit (BD Biosciences Clontech, Palo Alto, CA). AR-MC3T3 cultures were rinsed with serum-free media, stained with BD MitoSensor Dye at 37ºC for 20 min, and analyzed by fluorescence microscopy.

In vivo histological analysis

AR-transgenic mice with AR overexpression in the osteoblast lineage were employed for *in vivo* histological analysis. AR-transgenic mice, previously generated and characterized (59), were healthy and transmitted the transgene at the expected frequency. For experiments, hemizygous littermates were obtained by mating transgenic founders with B6D2F1 wild-type mice of both genders. Calvaria were isolated from AR-transgenic mice and littermate controls at 2 months and 6 months old, fixed in 4% paraformaldehyde, decalcified in Immunocal (Decal Corp., Tallman, NY), and then processed for paraffin embedding as previously described (59). 5-6 µm sections were cut and stained with hematoxylin and eosin (H&E). New bone growth was localized in calvarial cross sections using van Geison staining to identify collagen synthesis that has formed within 4 days of isolation (17). Apoptosis-induced DNA fragmentation in bone sections was assessed by means of Terminal-dUTP-Transferase-Nick-End Labeling (TUNEL) staining using TdT-FragEL DNA fragmentation detection kit (Oncogene research products, Boston, MA) following manufacturer's recommendations. Detection was performed using a streptavidin-horseradish peroxidase conjugate and 3,3'-diaminobenzidine as a color substrate. Sections were counterstained with methyl green,

mounted, and viewed under a microscope. Apoptotic cells are identified with condensed nuclei and dyed brown. Positive and negative controls were always included. Positive control sections were treated with 1 µg/µl DNase I in TBS with 1 mMgSO$_4$ for 20 min at room temperature following proteinase K treatment. Negative control sections were incubated without TdT.

Transient transfection, overexpression and RNA interference (RNAi) analysis

Experiments were performed to alter Bax/Bcl-2 ratio by transient transfections. Bcl-2 overexpression was achieved using pCMV-Bcl-2 expression construct (kindly provided by Dr. Stanley Korsmeyer, Harvard Medical School). AR-MC3T3 cells were plated onto 6-well dish and transfected with 2 µg of DNA per well using FuGENE 6 transfection (Roche, Basel, Switzerland) reagent at a 6:1 ratio (FuGene6: µg DNA). To inhibit *bax* gene expression, an oligonucleotide-based technique with double-stranded short interfering RNAs (siRNA) validated for *bax* was used, following manufacturer's instructions. Bax siRNA was introduced via transient transfection with SureSilencing Mouse Bax siRNA Kit (SuperArray Bioscience Corp., Frederick, MD) and Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). Transfections included negative control siRNA populations. AR-MC3T3 cells were transfected with indicated sequences (pCMV-Bcl-2 or Bax siRNA). Cultures were exposed to 10^{-8} M DHT for 30 h followed by 18 h-treatment with 50 µM etoposide and then isolated at day 5. Apoptosis was evaluated using the Cell Death oligonucleosome assay in transfected and mock-transfected cultures. The data are expressed as the mean ± SEM. Confirmation of alteration of protein levels of Bcl-2 and Bax was determined with Western blotting (see Fig. 6).

Statistical analysis

All data were analyzed using Prism v4.0 software (GraphPad Software, Inc.; San Diego, CA). Values from duplicate or triplicate wells from two to three experiments were used for statistical analysis with Student's *t* test or one-way ANOVA followed by post-hoc analysis with Newman-Keuls

Multiple Comparison Test. Differences of $P < 0.05$ were considered statistically significant. Results

are presented as the mean $\pm$ SEM.

RESULTS

Model of enhanced androgen responsiveness

AR levels are low as osteoblasts proliferate but increase during differentiation *in vitro* to reach maximal levels in mature osteocytic-like cells (55). In order to enhance androgen responsiveness, particularly in early proliferating osteoblasts, we have employed cultures of the immortalized mouse calvarial osteoblastic cell line MC3T3-E1 stably transfected with an AR expression construct under the control of 3.6 kb of the rat α1(I)-collagen promoter (for details, see reference 57). AR-MC3T3 cells were used as stable pools and grown under selection with G418.

To determine total AR levels throughout *in vitro* differentiation, both proliferating and osteocytic AR-MC3T3 cultures were characterized with AR binding and Scatchard plot analyses (Table 1). As can be seen, there is an approximately 3-fold elevation in AR binding between control ßgal-MC3T3 vs. AR-MC3T3 cultures in proliferating cultures at day 5. In mature osteoblastic/osteocytic cultures at day 25, there is also approximately 3-fold elevation in AR levels compared to control cultures. In addition, AR levels nearly doubled during differentiation in AR-MC3T3 cultures, with a less robust increase in control cultures. Analysis by two-way ANOVA demonstrated that both cell line and time in culture significantly influenced AR levels. AR protein abundance was also determined by Western analysis in whole cell lysates isolated during proliferation (day 5) and at a more mature osteoblast stage (day 20). As shown in Fig. 1A, total AR levels (from both endogenous AR and colAR transgene expression) are elevated in mature cultures. We next analyzed expression of the colAR transgene by qRT-PCR analysis using total RNA isolated from AR-MC3T3 cultures with primers specific for the colAR transgene or for endogenous AR (Fig. 1B). AR-transgene expression was elevated approximately 1.7-fold relative to the endogenous AR gene.

In order to evaluate whether overexpression of AR in these cultures alters steroid receptor signaling in a generalized non-specific fashion, we characterized transactivation mediated by a distinct steroid receptor, i.e., estrogen transactivation of ERs in AR-MC3T3 cultures. ER activity was evaluated in transient transfections using the ERE reporter construct ERE-luc. Cells were treated

for 54 h with E_2 in a dose response (10^{-12} M - 10^{-8} M) in the presence or absence of 10^{-8} M DHT. AR-MC3T3 cultures demonstrated a dose-dependent stimulation in ER transactivation with approximately 1.6 fold elevation with 10^{-8} M E_2. Importantly, E_2 signaling was not altered during coincident AR transactivation by co-treatment with combined 10^{-8} M E_2 and DHT, nor was DHT alone sufficient to activate ERE-dependent ER transactivation (Fig. 1C).

Characterization of osteoblast apoptosis: Androgen and estrogen effects during proliferation

We have previously shown that continuous DHT treatment reduces osteoblast viability through reductions in MAP kinase signaling in both normal rat primary calvarial cells and in AR-MC3T3 cultures (57). Since reductions in viability can be associated with enhanced apoptosis, we sought to determine whether DHT treatment also influenced osteoblast apoptosis. Three independent assays were employed to characterize apoptosis: nucleosome fragmentation, caspase-3 activity and $\Delta \Psi_{mito}$ analysis. AR-MC3T3 cells were plated in 6-well dishes and grown for 5 days in the continuous presence of either vehicle, 10^{-8} M DHT or 10^{-8} M E_2. Apoptosis was induced by treatment with 50μM etoposide for 18 h before harvest then assessed by oligonucleosome analysis (Fig. 2A) and with determination of caspase-3/CPP32 activity measured by substrate cleavage (Fig. 2B). DHT treatment significantly enhanced osteoblast apoptosis 1.5 to 2-fold, in the range described for well-characterized proapoptotic effects of glucocorticoid or TNFα(see 2, 45) and with BMP-2 administration (see 16) in osteoblasts. Separate one-way ANOVAs for each treatment determined that both androgen and estrogen treatments were significantly different from control but with opposite effects: DHT treatment significantly enhanced while E_2 treatment inhibited apoptosis. Although not as robust presumably due to lower AR levels, DHT also stimulated apoptosis in control ßgal-MC3T3 cultures (data not shown).

To extend these results, mitochondrial membrane permeability $\Delta \Psi_{mito}$ analysis was performed. AR-MC3T3 cells were rinsed with serum-free media, stained with MitoSensor Dye at 37ºC for 20 min, and analyzed by fluorescence microscopy (Fig. 2C). Collapse of the electrochemical gradient

across the mitochondrial membrane is an early indicator of the initiation of cellular apoptosis. MitoSensor (cationic dye) is taken up in the mitochondria where it forms red fluorescent aggregates in healthy cells. In apoptotic cells, the dye cannot aggregate because of altered $\Delta\Psi_{mito}$ and remains in a green fluorescent monomeric form in the cytoplasm. Modestly increased green/yellow staining with etoposide, enhanced with DHT treatment, is consistent with loss of mitochondrial membrane permeability and $\Delta\Psi$. In contrast, red stained cells with E_2 treatment suggests cultures are relatively protected from etoposide-induced apoptosis. In control cultures without etoposide induction, DHT and E_2 demonstrated similar but even less robust effects.

To establish whether androgen-induced apoptosis was mediated by functional AR, AR-MC3T3 cultures were treated with DHT in the presence of the specific non-steroidal AR antagonist hydroxyflutamide (OHF). As shown in Fig. 2D, an ~2.2-fold increase in apoptosis is observed after 10^{-8} M DHT treatment for 5 days ($P < 0.01$). However, coincubation with of 10^{-6} M OHF significantly abrogated the effect of DHT on apoptosis ($P < 0.05$). OHF alone appeared to have a slight agonist effect as has been noted previously (see 56), however the effect was not significantly different from control.

Characterization of osteoblast apoptosis: Androgen effects during differentiation in mature osteoblast/osteocyte cultures

Since much of the analysis of the effects of estrogen on osteoblast apoptosis has focused on osteocytic cells, known to demonstrate enhanced apoptosis (31), we next determined whether the effect of either estrogen or androgen treatment on osteocytic cells in extended culture at day 29 was similar to that observed in proliferating osteoblasts. We have shown that both ERα and ERβ mRNA and protein expression in osteoblastic cells was similar or elevated at day 25 or 30 when compared with day 5 (55), and that AR-MC3T3 osteocytic cultures have enhanced AR expression (Fig. 1 and Table 1). Osteocytic cultures were characterized after continuous DHT and E_2 treatment (both at 10^{-8} M) treatment for 29 days (Fig. 3). Cultures were serum starved for the last 48 h in medium

containing 0.1% bovine serum albumin. Apoptosis was induced in osteocytic cultures by serum

starvation instead of etoposide administration since topoisomerase II activity (the target of etoposide

treatment) is dramatically reduced in highly confluent cultures (47). Quantitative analysis of

apoptosis was determined using nucleosome fragmentation, caspase-3 activity and mitochondrial

membrane permeability as described in Fig. 2. Consistent with results in proliferating cultures, DHT

enhanced but E_2 inhibited osteoblast apoptosis after continuous treatment in day 29 osteocytic

cultures assessed by oligonucleosome analysis (Fig. 3A) and loss of mitochondrial membrane

permeability (Fig. 3C). DHT treatment modestly increased caspase-3 activity but this result did not

reach significance (Fig. 3B); however E_2 did not suppress caspase-3 activity. These results may

reflect the high levels of caspase-3 expression in osteocytic cultures *in vitro* (35).

DHT-mediated changes in key apoptotic regulators Bcl-2 and Bax

In order to characterize the mechanism underlying DHT-enhanced osteoblast apoptosis, we next

determined the effect of androgen treatment on the important apoptotic regulators Bcl-2 and Bax.

Western blot analysis was used to determine the levels of Bcl-2 and Bax protein in DHT- or E_2-

treated AR-MC3T3 cells (Fig. 4A). After DHT treatment, we observed significant enhancement of

the Bax/Bcl-2 ratio by nearly 1.3-fold (P < 0.05; Fig. 4B), which mostly reflected a decrease in Bcl-2

protein levels. E_2 treatment alone did not significantly alter the Bax/Bcl-2 ratio, consistent with

previous analysis in osteoblasts (12). To better characterize the time course of changes in Bcl-2

protein, Western analysis was performed after 24, 48, 72 and 96 h of continuous 10^{-8} M DHT

treatment (Fig. 4C). Quantitative analysis of Bcl-2 protein levels indicated by fold change showed

that Bcl-2 levels were relatively unaffected for up to 48 h of DHT treatment, but then decreased by

72 h and 96 h of treatment to ~70% of control levels. This result is consistent with the time course

observed for androgen-mediated changes in osteoblast viability (57).

We next investigated the mechanism by which androgen treatment stimulated the proapoptotic

response by determining *bcl-2* and *bax* steady-state mRNA levels with real time qRT-PCR analysis.

AR-MC3T3 cells were incubated continuously with either DHT or E_2 (both at 10^{-8} M) for 5 days and total RNA was isolated. Fig. 4D shows that *bax* mRNA levels were modestly elevated ~20% with DHT treatment, although this effect did not reach statistical significance. In contrast, *bcl-2* mRNA levels were not different compared to those for control vehicle-treated cells. Given the reductions in Bcl-2 protein but no corresponding decrease in *bcl-2* mRNA levels, these results suggest that DHT regulation of Bcl-2 protein levels is post-transcriptional.

The androgen-mediated reduction of Bcl-2 protein is associated with decreased Bcl-2 serine phosphorylation and increased ubiquitination

MAP kinase activity has been shown to influence Bcl-2 protein degradation through changes in Bcl-2 phosphorylation (5, 9). Since we have previously demonstrated reduced MAP kinase signaling after chronic androgen treatment in osteoblastic cells (57), we sought to determine whether androgen treatment in osteoblasts also reduced Bcl-2 phosphorylation. AR-MC3T3 cells were treated with 10^{-8} M DHT for 3 days and whole cell extracts were prepared. Equal amounts of protein were analyzed for the presence of total and phosphorylated Bcl-2 after immunoprecipitation followed with Western blot analysis using either pan Bcl-2 or anti-phosphoserine-specific antibody (clone 4A9) for detection (Fig. 5A, *left panel*). DHT treatment reduced Bcl-2 protein to ~80% of control levels, with an additional reduction in serine phosphorylation to ~70% of normal levels (Fig. 5A, *right panel*). To provide further evidence that proteasomes were involved in Bcl-2 degradation after androgen treatment, AR-MC3T3 cells were treated with DHT and the effect on ubiquitination of Bcl-2 was determined. Total Bcl-2 was isolated from whole cell lysates by immunoprecipitation followed by Western blot analysis with anti-ubiquitin antibody. As shown in Fig. 5B, ubiquitin conjugates of Bcl-2 were modestly increased by DHT treatment, consistent with post-transcriptional regulation. Combined, these results suggest that targeting of Bcl-2 for degradation by proteasomes is associated with reduced serine phosphorylation of Bcl-2 protein.

The apoptotic effect of DHT requires an increase in Bax/Bcl-2 ratio

In order to determine whether apoptosis stimulated by androgen treatment requires an increase in Bax/Bcl-2 ratio, expression of either *bcl-2* or *bax* mRNA was altered by transiently transfection. To reverse the inhibition of Bcl-2 expression by androgen treatment, Bcl-2 was overexpressed using the pCMV-Bcl-2 expression construct. AR-MC3T3 cells were transfected with pCMV-Bcl-2 or mock-transfected, then treated with DHT for a total of 48 h (30 h followed by 50 µM etoposide for 18 h). Apoptosis was characterized at day 5 using mono- and oligonucleosome analysis as described in Fig. 2. In osteoblastic cultures with Bcl-2 overexpression (Fig. 6A), androgen-enhancement of apoptosis was completely reversed ($P < 0.001$), confirming the importance of reductions in Bcl-2 levels. Overexpression of Bcl-2 protein was confirmed by Western analysis (Fig. 6C, *left panel*).

We next employed RNAi to inhibit Bax protein using an oligonucleotide-based technique with double-stranded siRNA validated for *bax*. AR-MC3T3 cells were transfected with siRNA Bax-specific oligonucleotides 24 h prior to the exposure of cells to DHT for 30 h, followed by 50µM etoposide for 18 h before isolation at day 5. As shown in Fig. 6B, DHT-mediated increases in osteoblast apoptosis were significantly abrogated with Bax inhibition ($P < 0.001$). Western analysis of Bax protein levels in androgen treated AR-MC3T3 cells expressing Bax siRNA (Fig. 6C, *right panel*) confirmed that siRNA treatment resulted in a reduction in Bax protein expression to 60% of normal levels, with no effect after transfection with control siRNA oligonucleotides. A second source of *bax* siRNA oligonucleotides also abrogated DHT-mediated increases in osteoblast apoptosis (data not shown). These data suggest that an increase in the Bax/Bcl-2 ratio is important for androgen-mediated apoptosis, and is consistent with reports that Bcl-2 and Bax can function independently to regulate apoptosis (26).

In vivo stimulation of osteoblast apoptosis in AR-transgenic mice

In order to assess the physiological relevance of these observations, we characterized apoptosis in skeletally targeted AR-transgenic mice (for a description of the phenotype observed in these

animals, please refer to reference 59). AR-transgenic mice were created with the same full-length AR expression construct used for the AR-MC3T3 cells, demonstrate AR overexpression throughout the osteoblast lineage, and represent a model of enhanced responsiveness to circulating androgen without systemic hormone administration. An anabolic response to androgen in males was observed in H&E stained sections from calvaria isolated from male AR-transgenic mice vs. littermate controls at 2 months (Fig. 7A, *left panels*) and 6 months (Fig. 7A, *right panels*), and was not observed in females as previously reported (59). Localization of new bone growth was characterized in calvarial cross sections using van Geison staining, where recently formed collagen is identified by a characteristic blue color (17). As can be seen in Fig. 7B, new bone growth was present in calvaria as a result of AR-transactivation in post-pubertal (i.e., 2 month old; *left panel*) and adult (i.e., 6 month old; *right panel*) male AR-transgenic mice. There may be increased cell density in the woven bone present in the AR-transgenic male calvaria, consistent with the notion that woven bone has been estimated to contain 4 to 8 times more osteocytes than lamellar bone (18).

Previously, characterization of the effects of androgen treatment on osteoblast apoptosis *in vivo* have been only performed in recently orchidectomized animals in a setting of high turnover and activated resorption (27), rather than associated with bone growth in an anabolic circumstance. We therefore characterized apoptosis in male AR-transgenic animals with TUNEL staining in calvarial cross sections. Post-pubertal (Fig. 7C, *left panels*) and adult (Fig. 7C, *right panels*) male AR-transgenic mice showed increased TUNEL staining when compared to wild-type controls, both in areas of new bone growth (proliferating osteoblasts). TUNEL staining was also demonstrated in matrix-embedded osteocytes in calvarial sections where new bone growth was not evident (Fig. 7D). Negative controls verified a lack of staining (see *inset*). Higher power magnifications of the apoptotic cells (see *insets*) identify the apoptotic cells as osteoblastic and not multi-nucleated osteoclasts, based on location and morphology.

DISCUSSION

In this study we explored the specific role of androgen, contrasted with estrogen, in the regulation of osteoblast apoptosis using *in vivo* and *in vitro* models of enhanced androgen responsiveness. We have previously demonstrated that chronic androgen treatment reduces osteoblast viability in both normal primary cultures and in clonal colAR-MC3T3 cultures, an effect mediated via inhibition of the ERK cascade (57). Consistent with those findings, we report here that continuous treatment with the non-aromatizable androgen DHT stimulated, while similar treatment with E_2 reduced, osteoblast apoptosis. Androgen treatment enhanced osteoblast apoptosis in both proliferating and mature osteocyte-like cells *in vitro*, and the effects were abrogated in the presence of an androgen receptor antagonist. Levels of the antiapoptotic regulator Bcl-2 were reduced post-transcriptionally by androgen treatment, associated with enhanced ubiquitination. Reduced serine phosphorylation of Bcl-2 was also observed, consistent with inhibition of ERK1/2 activity. Notably, the increased Bax/Bcl-2 ratio was necessary for androgen-enhanced apoptosis since overexpression of *bcl-2* or siRNA knockdown of *bax* abrogated the effects of DHT. *In vivo* analysis of calvarial sections from skeletally targeted male AR-transgenic mice at two time points also demonstrated enhanced TUNEL staining in osteoblasts when compared with wild-type controls, even in areas of new bone growth. Collectively these data indicate that enhanced osteoblast apoptosis after androgen exposure is mediated by an increase in the Bax/Bcl-2 ratio via a reduction in Bcl-2 phosphorylation and MAP kinase activation, and is observed even in anabolic settings.

Notably, an association of osteoblast apoptosis with osteogenesis has been observed previously. During bone formation, osteoblasts undergo an orderly developmental progression ultimately ending in apoptosis (19). It has been estimated that as many as 65% of osteoblasts undergo apoptosis after completing synthesis of bone matrix (25), suggesting that apoptosis is a fundamental component of the osteoblastic differentiation program (31). TUNEL-positive osteoblasts and even preosteoblasts have been demonstrated at or close to the osteogenic fronts in areas of intense osteogenic activity during calvarial growth (40). In addition, caspase-3-deficient mice show delayed

ossification and decreased bone mineral density, suggesting that caspase activity plays a critical role in osteogenic differentiation *in vivo* (34). Finally, bone-targeted Bcl-2 overexpression leads to reduced bone formation in a sex-specific fashion (38). Thus, apoptosis can be associated with osteogenesis and the homeostatic maintenance of bone. This point is worth noting, since androgen-mediated apoptosis might be inappropriately viewed as inconsistent with anabolic actions.

In some tissues, androgen treatment has been reported to enhance apoptosis (54, 64) and decrease Bcl-2 levels (6, 20, 30). In this report we show that treatment with DHT in osteoblasts also results in reduced Bcl-2 protein levels and enhanced apoptosis. Bcl-2 protein can be stabilized upon ERK1/2 activation in some settings (8, 14, 41). While it has been reported that Bcl-2 phosphorylation can also facilitate apoptosis (60), it may also be that Bcl-2 phosphorylation can enhance survival but is not sufficient to prevent cell death with continuous toxin exposure (8). Consistent with our studies, dephosphorylation of Bcl-2 results in enhanced apoptosis, mediated by ubiquitin-induced degradation of Bcl-2 (5, 9). These reports suggest a link between MAP kinase activity and the proteasome pathway to regulate Bcl-2 levels. Although three consensus MAP kinase sites have been identified in Bcl-2 (5), Bcl-2 is the target of multiple kinases including c-Jun N-terminal kinase 1, protein kinase A and PKCα (8, 23). Furthermore, Bcl-2 phosphorylation is a dynamic process that is negatively regulated by protein phosphatase 2A (21). In addition, the antiapoptotic effect of growth factor signaling may be mediated by additional proteins through PI3 kinase-mediated pathways (53). Thus, it is likely that the regulation of the antiapoptotic function of Bcl-2 by phosphorylation is cell-type specific and may be dependent on additional factors that are as yet undefined. Here we demonstrate that in osteoblasts, androgen treatment decreases Bcl-2 protein levels post-transcriptionally with reduced serine phosphorylation and increased ubiquitination. Thus, one mechanism through which androgen treatment enhances osteoblast apoptosis may be via inactivation of the antiapoptotic function of Bcl-2 by reducing Bcl-2 protein level through ubiquitin-mediated proteolytic degradation, as expected with suppressed ERK1/2 activity (57).

The antiapoptotic effect of estrogen and the proapoptotic effect of androgen treatment were observed both in proliferating osteoblasts and in mature osteocytic cultures. These results are supported by a recent publication of opposing effects of estrogen and androgen on apoptosis, here in T47D cells (22). Further, our *in vitro* analyses are consistent with multiple reports indicating that estrogen can reduce osteoblast apoptosis in some (12, 27, 65), but not all circumstances (11). Neither Bcl-2 nor Bax expression was significantly influenced by E_2 treatment alone, contrary to the effects of androgen treatment. In the presence of apoptotic stimuli such as glucocorticoid treatment, E_2 is able to prevent the increased Bax/Bcl-2 ratio (12, 13, 38). To our knowledge, this is the first report demonstrating divergent and distinct regulation by androgen vs. estrogen with regard to osteoblast apoptosis. These results again indicate that non-specific non-genomic signaling through either ERs or AR, reportedly activated with the same effectiveness regardless of whether the actual ligand is an androgen or an estrogen (27), is not a significant modulator of osteoblast apoptosis induced by androgen treatment. Analysis with AR overexpression in Fig. 1C is also consistent with mediation of androgen action strictly by AR transactivation, and not through non-specific interactions with ERs nor through generalized squelching of steroid receptor mediated signaling.

A contrasting response to estrogen and androgen administration has also been described in some bone compartments *in vivo*. This is particularly true in cortical bone, where at the periosteum estrogen suppresses but androgen stimulates new bone formation (49), while at the endosteum estrogen stimulates but androgen strongly suppresses formation (59). These envelope-specific responses likely play an important role in determining sexual dimorphism of the skeleton, i.e. that male bones are wider but not thicker than females (43). In trabecular bone, ER and AR signaling may have functional redundancy, although not with exactly the same mechanism (36). Combined, these results suggest that administration of both estrogen and androgen may be beneficial, and is consistent with clinical studies demonstrating that combination therapy with estrogen and androgen is more beneficial than either steroid alone in post-menopausal women (7, 33), recently confirmed in

an animal model (46). Ongoing analysis examining the effects of both steroids in combination will be important for a better understanding of the impact of combination therapy on bone homeostasis.

Few studies have characterized the specific effect of androgen *in vivo* on osteoblast apoptosis. In one report, increased osteoblast apoptosis was seen after orchidectomy in adult mice (where both estrogen and androgen levels are reduced). Six weeks of DHT treatment in these orchidectomized mice was associated with reduced osteoblast apoptosis in vertebrae (27), a site of predominantly trabecular bone. However in this setting soon after orchidectomy, bone resorption is dramatically increased and thus one cannot adequately evaluate a connection between anabolic effects of androgen treatment associated with new bone growth and osteoblast apoptosis. Results reported here are also in contrast to the *in vitro* response noted after short-term DHT treatment that demonstrated inhibition of apoptosis (27). While the reasons for the discrepancy are not clear, inhibition of apoptosis reported in this setting may have been a consequence of stimulation of ERK activity associated with rapid non-genomic steroid actions, since longer androgen treatment results in reductions in MAP kinase activation (57). Interestingly, it is possible that some aspects of the anabolic response to androgen we and others have described could be the result of an increase in osteoblast cell proliferation via a transient non-genomic androgen-mediated increased MAP kinase, PKC or PI3 kinase or other kinase cascades and/or calcium mobilization (e.g., see 24), through potentiation of growth factor and/or cytokine signaling, or through an as yet unexplained mechanism. Androgen treatment has been reported to increase osteoblast proliferation *in vitro* when treatment times are short (57) or transient (e.g., 15 minutes, see 24), and to increase collagen expression (3). The anabolic response is likely to be complicated however, since there is no clear mechanism to block androgen signaling after transient induction *in vivo.* In addition, osteogenesis *in vivo* is generally not associated with estrogen treatment that does increase MAP kinase activity (10).

In conclusion, we have demonstrated that chronic DHT treatment in both proliferating and in mature osteocytic cultures resulted in enhanced osteoblast apoptosis. This result contrasts with the inhibitory effects on apoptosis observed with E_2 treatment. An androgen-mediated increase in the

Bax/Bcl-2 ratio was observed, predominantly through inhibition of Bcl-2. Increased Bax/Bcl-2 was necessary and sufficient for androgen-enhanced apoptosis since overexpression of *bcl-2* or RNAi knockdown of *bax* abrogated the effects of DHT, and was dependent on functional AR. These data suggest that enhanced apoptosis is mediated by an increase in the Bax/Bcl-2 ratio, at least in part as a consequence of reductions in Bcl-2 phosphorylation and protein stability consistent with inhibition of MAP kinase pathway activation. Analysis of calvaria in AR-transgenic male mice demonstrated enhanced TUNEL staining *in vivo* even in areas of new bone growth. In bone, apoptosis in osteoblasts has been reported *in vitro* and during development *in vivo* (31). As has been observed in other remodeling tissues and/or associated with development and tissue homeostasis (29), mounting evidence has identified an association between new bone growth and apoptosis (37). Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density (34). Collectively, the findings reported here help to resolve a controversy surrounding the role of sex steroids in bone homeostasis by demonstrating that androgen directly stimulates while estrogen inhibits osteoblast apoptosis, and suggests that modulation of osteoblast apoptosis may be an important consequence of both androgen and estrogen signaling but with distinct outcomes. These data demonstrate that androgen signaling through the AR in bone directly influences osteoblast function in growing tissue *in vivo* and offer valuable insight into the role of androgen signaling in bone homeostasis. Further studies on androgen action in the developing skeleton, during repair and in the adult are warranted.

Acknowledgments

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Table 1. Analysis of AR levels with differentiation in AR-MC3T3 cultures

Tissue/Cells	Kd x 10^{-10} M	Bmax (fmol/mg DNA)	Capacity (sites/cell)
d5 ßgal-MC3T3	1.9 ± 0.45	644 ± 138	2323 ± 496
d5 AR-MC3T3	2.2 ± 0.05	1491 ± 16	5383 ± 60
d15 ßgal-MC3T3	1.5 ± 0.03	991 ± 160	3576 ± 576
d15 AR-MC3T3	2.9 ± 0.60	1446 ± 86	5219 ± 311
d25 ßgal-MC3T3	1.8 ± 0.20	1308 ± 469	4720 ± 1691
d25 AR-MC3T3	2.6 ± 0.20	2479 ± 405	8947 ± 1461
Ventral Prostate	5.8 ± 0.06	1351 ± 85	4877 ± 308
LNCaP	7.7 ± 1.57	3971 ± 598	14335 ± 2157

Androgen receptor levels were quantitated by titration analysis using [^{3}H]methyltrienolone ([^{3}H]R1881). The DNA content was quantitated and specific [^{3}H]R1881 binding capacity was estimated by one site binding with non-linear regression and Scatchard plot analysis. The specific binding of [^{3}H]R1881 was expressed in fmol/mg DNA and sites/cell. Analysis by two-way ANOVA demonstrated that both cell line and time in culture significantly influenced AR levels.

Figures

Fig. 1 Characterization of AR levels in AR-MC3T3 cultures during osteoblast differentiation.

AR levels in AR-MC3T3 cultures during proliferation (day 5) and in mature osteoblasts/osteocytes

(day 20) were assessed by Western analysis. **A. AR protein levels in stably transfected AR-**

MC3T3 and control ßgal-MC3T3 cells. Both AR-MC3T3 and control ßgal-MC3T3 cells were grown

for the indicated time in culture, and AR abundance was determined in whole cell lysates with

Western blot analysis using polyclonal rabbit antibodies recognizing AR (PA1-111A). The same

membrane was reprobed with α-tubulin antibodies as a loading control. The increase in AR levels

with differentiation is also seen by Scatchard plot analysis presented in Table 1. **B.**

Characterization of colAR transgene gene expression by qRT-PCR analysis.   Primers specific

for the colAR transgene and for endogenous AR were used to evaluate relative AR transgene

expression using total RNA isolated from AR-MC3T3 cultures. **C**. **Lack of effect of AR**

overexpression on transactivation of other steroid receptors. ER function was evaluated in AR-

MC3T3 cultures in transient transfections using the ERE reporter construct ERE-luc. Cells were

treated for 54 h with E_2 at 10^{-12} M, 10^{-10} M and 10^{-8} M in the presence or absence of 10^{-8} M DHT. The

data are expressed as the means $\pm$ SEM (n = 6) from two independent experiments. Analysis by

ANOVA revealed significant differences ($P < 0.0001$). Post-hoc analysis using Newman-Keuls

Multiple Comparison Test demonstrated a significant increase in reporter activity with E_2 treatment,

but no effect of DHT alone or in the presence of E_2. *, $P < 0.05$; ***, $P < 0.001$ vs control; #, $P <$

0.001 vs. E_2 + DHT combined.

Fig. 2 Characterization of osteoblast apoptosis: Results of androgen and estrogen treatment

during proliferation. AR-MC3T3 cells were plated in 6-well dishes and grown for 5 days in the

continuous presence of vehicle or 10^{-8} M DHT or 10^{-8} M E_2. Apoptosis was induced by treatment

with 50µM etoposide for 18 h before harvest. Apoptosis was characterized using three independent

assays. **A. Cell Death oligonucleosome analysis**. Apoptosis was characterized by analyzing

DNA fragmentation with the Cell Death oligonucleosome assay. The data are presented as the

nucleosome enrichment factor = A $_{treated}$/A $_{untreated}$, expressed as the means $\pm$ SEM (n = 9-12) from

three separate studies. Differences were statistically significant by student's t test. **, $P < 0.01$; ***,

$P < 0.0001$ vs. control. **B. Caspase-3/CPP32 activity measured by substrate cleavage**. Data

are presented as mean $\pm$ SEM (n = 8). Experiments were carried out three times. *, $P < 0.05$; **, $P <$

0.01 vs. control. **C. Mitochondrial membrane potential ($\Delta\Psi_{mito}$) analysis**. AR-MC3T3 cells were

rinsed with serum-free media, stained with MitoSensor Dye at 37°C for 20 min, and analyzed by

fluorescence microscopy. All results are representative of at least three independent experiments.

D. Characterization of osteoblast apoptosis: Dependence on AR function. AR-MC3T3 cells

were cultured as described with 10^{-8} M DHT treatment for 5 days with or without 10^{-6} M

hydroxyflutamide (OHF), an androgen receptor antagonist. Apoptosis was evaluated by Cell Death

oligonucleosome analysis. The data are expressed as the means $\pm$ SEM (n = 6) from two

independent experiments. Differences were statistically significant by one-way ANOVA (P = 0.0033).

Post-hoc analysis with Newman-Keuls Multiple Comparison Test demonstrated that

hydroxyflutamide significantly abrogates DHT-mediated apoptosis at day 5. **, $P < 0.01$ (vs. control);

#, $P < 0.05$ (vs. DHT).

**Fig. 3 Characterization of osteoblast apoptosis: Androgen effects during differentiation in

mature osteoblast/osteocyte cultures.** Apoptosis was characterized in osteocyte-like cultures

after continuous DHT and E$_2$ treatment (both at 10^{-8} M) for 29 days. Apoptosis was induced by

serum starvation for 48 h before isolation, replaced with 0.1% BSA. **A. Analysis of apoptosis

evaluating DNA fragmentation analysis**. The data are expressed as the means $\pm$ SEM (n = 6)

from two independent experiments. See Fig. 2 for methods. **, $P < 0.01$ vs. control. **B. Caspase-

3/CPP-32 activity measured by substrate cleavage**. Lysates from day 29 control and hormone-

treated cultures were analyzed. The data are expressed as the means $\pm$ SEM (n = 6) from two

independent experiments. **C. Mitochondrial membrane potential ($\Delta\Psi_{mito}$) analysis**. AR-MC3T3

cells were cultured with continuous DHT and E_2 treatment (both at 10^{-8} M) for 29 days, stained with MitoSensor Dye, and analyzed by fluorescence microscopy. All results are representative of at least three independent experiments.

Fig. 4 DHT-mediated changes in key apoptotic regulators Bcl-2 and Bax. AR-MC3T3 cells were isolated at day 5 after continuous DHT or E_2 (10^{-8}M) treatment.   **A. Western blot analysis of Bcl-2 and Bax levels.** Total cell lysates from AR-MC3T3 treated with steroids for 5 days were used to evaluate Bcl-2 and Bax protein levels. Extract was analyzed by 15% SDS-PAGE, transferred onto an Immobilon-P PVDF membrane and probed using polyclonal rabbit antibodies recognizing Bcl-2 or Bax as described in methods. Fold difference between control and DHT treatment is indicated for Bcl-2 levels. **B. Quantitative analysis of Bax/Bcl-2 ratio.** Quantification of immunoband intensities from Western blot analysis as shown in panel A was determined by densitometric scanning. Data were expressed as Bax/Bcl-2 ratio. Results are representative of at least three independent experiments. *, $P < 0.05$ vs. control. **C. Time course of Bcl-2 protein levels after androgen treatment.** AR-MC3T3 cultures were treated with 10^{-8}M DHT for 24, 48, 72 and 96h, and Bcl-2 levels were characterized by Western blot analysis.   Fold difference between control and DHT treatment is indicated for Bcl-2 levels. **D. Regulation of Bcl-2 is post-transcriptional**. Levels of *bcl-2* and *bax* mRNA were analyzed by qRT-PCR analysis from total RNA isolated from control, E_2 or DHT-treated AR-MC3T3 cultures for 5 days. Levels of *bcl-2* were unaffected by hormone treatment, while the levels of *bax* were modestly increased by DHT. The data are expressed as the means $\pm$ SEM (n = 4) from two experiments.

Fig. 5 The androgen-mediated reduction of Bcl-2 protein is associated with decreased Bcl-2 phosphorylation and proteasomal degradation. AR-MC3T3 cells were treated with 10^{-8} M DHT for 72 h and equal amounts of protein were immunoprecipitated with anti-Bcl-2 antibody. Immunocomplexes were resolved by 12% SDS-PAGE and transferred onto Immobilon-P PVDF

membrane. **A. Androgen treatment reduces Bcl-2 protein and induces Bcl-2**

dephosphorylation. Western blot (WB) analysis was carried out with either anti-Bcl-2 antibody or

anti-phosphoserine-specific antibody after immunoprecipitation (IP). Equal protein loading was

confirmed with α-tubulin levels determined before immunoprecipitation by Western analysis from the

same lysates. Quantification of immunoband intensities was determined by volumetric densitometric

scanning (*right* panel). The data are expressed as the means $\pm$ SEM (n = 4) from two independent

experiments. *, $P < 0.05$ vs. control. **B. Androgen induces Bcl-2 ubiquitination.** Ubiquitin

conjugates of Bcl-2 were identified with Western blot analysis using anti-ubiquitin antibody after

immunoprecipitation. conj., conjugates; Ig, the heavy chain of the immunoglobulin molecule.

Fig. 6 The proapoptotic effect of DHT requires a decline in Bcl-2/ Bax ratio. Bcl-2 or Bax levels

were altered in AR-MC3T3 cultures after transient transfection.   Cultures were transfected one day

after plating and exposed to 10^{-8} M DHT for 48 h. Apoptosis was induced by treatment with 50μM

etoposide for 18 h before isolation at day 5. **A. Effect of Bcl-2 overexpression.** Bcl-2 mRNA

overexpression was achieved by transfection with expression construct pCMV-Bcl-2. Apoptosis was

evaluated using the Cell Death oligonucleosome assay in transfected and mock-transfected

cultures. The data are expressed as the mean $\pm$ SEM (n = 6) from two experiments. Differences

were statistically significant by one-way ANOVA ($P < 0.0001$). Post-hoc analysis with Newman-

Keuls Multiple Comparison Test demonstrated that overexpression of Bcl-2 completely reverses

DHT-mediated apoptosis at day 5. ***, $P < 0.001$ (vs. control); #, $P < 0.001$ (vs. DHT control). **B.**

Effect of Bax knockdown with double-stranded siRNA treatment. Cultures were transfected

with control siRNA (con) or Bax siRNA (double-stranded Bax siRNA) using the validated

SureSilencing Mouse Bax siRNA kit as described in methods or were mock-transfected. Apoptosis

was evaluated using the Cell Death oligonucleosome assay in transfected and control cultures. The

data are expressed as the mean $\pm$ SEM (n = 6) from two experiments. Differences were statistically

significant by one-way ANOVA. Post-hoc analysis with Newman-Keuls Multiple Comparison Test

demonstrated that inhibition of Bax ameliorates DHT-mediated apoptosis at day 5. ***, $P < 0.001$ (vs. control); #, $P < 0.001$ (vs. DHT control).   **C. Western blot analysis for altered Bcl-2 and Bax expression.** Proteins from total cellular extracts isolated 48 h after mock transfection or cultures transfected with Bcl-2 expression plasmid (*left* panel) or control or Bax siRNAs (*right* panel) were analyzed by Western blot probed with Bcl-2 or Bax antibody. Equal protein loading was confirmed by analysis with α−tubulin antibody. Quantification of immunoband intensities was determined by densitometric scanning. The band intensity values of Bcl-2 or Bax were normalized with respect to the band intensity values of α-tubulin. -, mock-transfection; con, control siRNA oligonucleotides; +, bax siRNA oligonucleotides.

Fig. 7 Characterization of osteoblast apoptosis *in vivo* using AR-transgenic mice. Calvaria from either 2 month old (*left panels*) or 6 month old (*right panels*) male AR-transgenic or littermate control mice were fixed, decalcified, embedded and calvarial cross sections were evaluated. **A**. **Calvarial histology**. H & E stained cross sections show calvarial thickening in male AR-transgenic mice. Bar indicates 50µm. **B. New bone growth.** New bone growth associated with new collagen synthesis was identified in calvarial cross sections using van Geison staining. New collagen synthesis is shown in post-pubertal 2 month old mice and in adult 6 month old mice. **C**. **Analysis of apoptosis with TUNEL staining with new bone formation**. *In situ* end-labeling (TUNEL) staining was performed for analysis of apoptosis and counterstained with methyl green. Sections were derived from areas demonstrating new bone formation as described in C. Negative control sections for TUNEL analysis were incubated without TdT (*inset*). Quantitative analysis indicated ~10% apoptotic cells in wild-type and ~40% in AR-transgenic mice. **D**. **Analysis of apoptosis with TUNEL staining in osteocytic cells**. TUNEL staining was performed in calvarial outer regions that do not demonstrate thickening. Enhanced apoptosis was observed in male AR-transgenic mice both in areas with new bone formation and in matrix-embedded osteocytes. Higher power magnification

shows osteoblasts morphology (*inset*). wt, wild-type mice (*left panels*); tg, AR-transgenic mice (*right panels*). Microscope magnification at 40X with higher power inset image at 100X.

Figure 1

A

day 5   day 20

◀ AR

◀ α-tubulin

ß-gal   colAR   ß-gal   colAR

B

C

Figure 2

A

day 5

Nucleosome enrichment factor (fold/control)

con E$_2$ con DHT

** ***

B

day 5

Caspase-3/CPP32 activity (fold/control)

con E$_2$ con DHT

* **

C

Mitochondrial membrane potential

con E2 DHT

ETOP −

ETOP +

D

day 5

Nucleosome enrichment factor (fold/control)

con DHT D+OHF OHF

Treatments

** #

Figure 3

A

day 29

Nucleosome enrichment factor (fold/control)

con E$_2$ con DHT

B

day 29

Caspase-3/CPP32 activity (fold/control)

con E$_2$ con DHT

C

Mitochondrial membrane potential

con   E2   DHT

Serum

+

-

Figure 4

A

con   E₂   DHT

◄ Bax

◄ Bcl-2

◄ α-tubulin

fold   1.0   1.0   0.7

B

Bax/Bcl-2 (arbitrary units) vs **Treatments (10^{-8}M)**: con, E₂, DHT

C

24h   48h   72h   96h

◄ Bcl-2

◄ α-tubulin

DHT   −   +   −   +   −   +   −   +

fold   1.1   1.2   0.8   0.7

D

con, E2, DHT

mRNA/RiboGreen (fold/control) : bcl-2, bax

AR-MC3T3 at d5

Figure 5

A

B

Figure 6

A

Bcl-2

Bcl-2 overexpression

B

Bax

Bax knockdown

C

Western blot analysis

Figure 7

Appendix 3
Wiren KM, Toombs A, Zhang X-W: Enhanced apoptosis associated with anabolic actions of androgens in AR-transgenic mice: requirement of reduced bcl-2/bax ratio. (Abstract #M226) J Bone Miner Res, 2004

Enhanced Apoptosis Associated With Anabolic Actions Of Androgens In AR-Transgenic Mice: Requirement Of Reduced Bcl-2/Bax Ratio

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Like estrogen, non-aromatizable androgens have significant beneficial effects on skeletal homeostasis. Estrogen has been shown to protect osteocytes from apoptosis but the effects of androgen are still poorly understood. We demonstrated that prolonged androgen signaling leads to reduced osteoblast viability, mediated via inhibition of the extracellular signal regulated kinase (ERK) cascade in both primary cultures and in an osteoblastic model with enhanced androgen responsiveness: MC3T3-E1 cells stably transfected with androgen receptor (AR) under the control of the 3.6-kb α1(I)-collagen promoter (colAR-MC3T3). Based on this evidence, we sought to elucidate the effects of 5α-dihydrotestosterone (DHT) on osteoblast apoptosis in colAR-MC3T3 cultures. In addition, recently characterized AR-transgenic mice with AR overexpression in the osteoblast lineage provide an enhanced model to examine in viv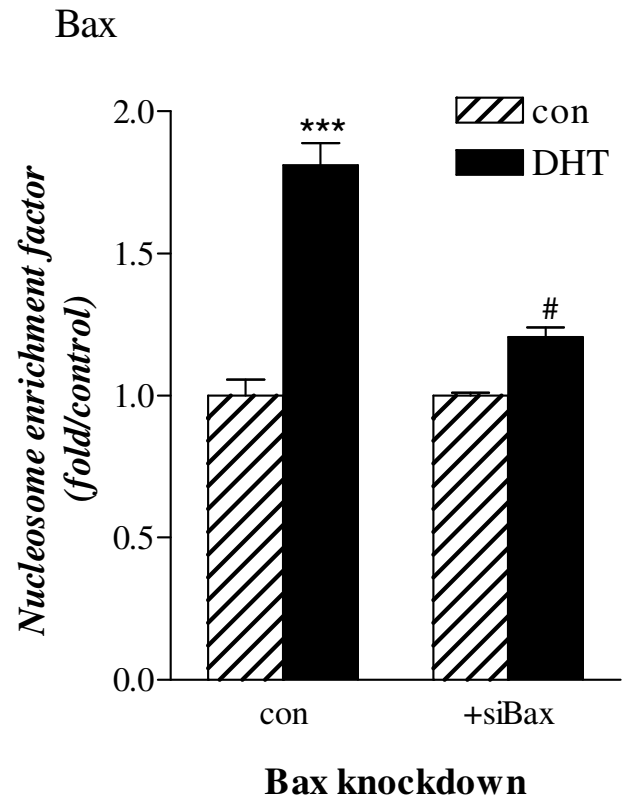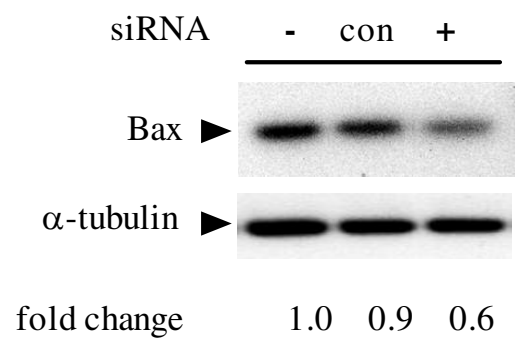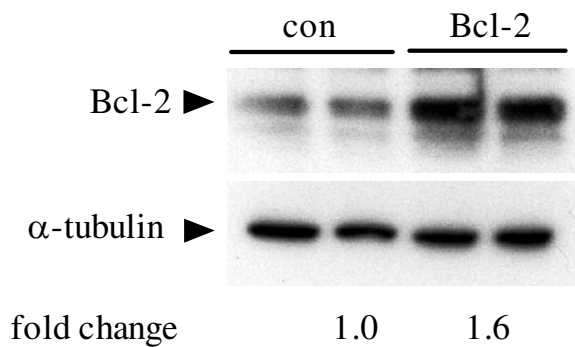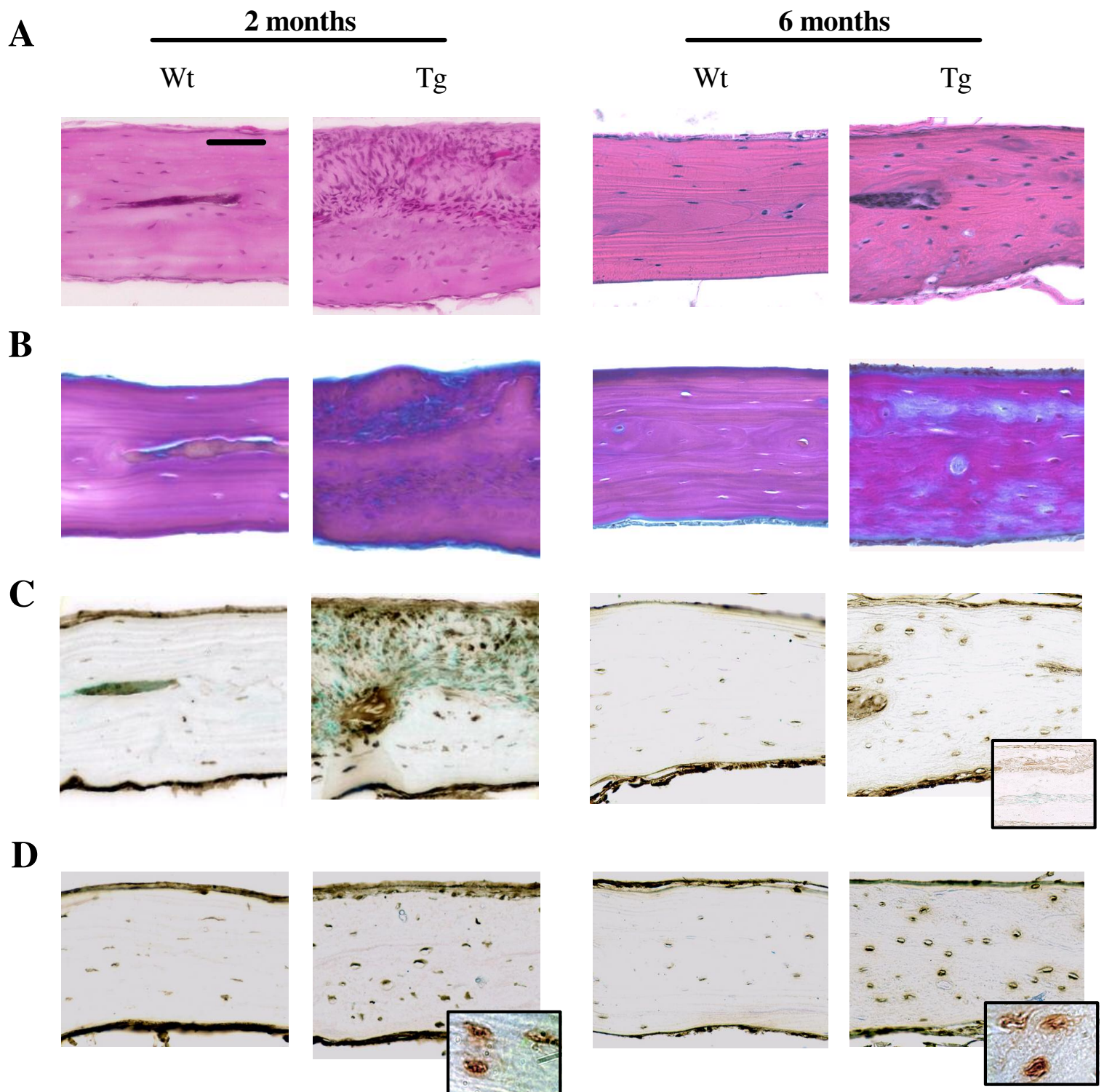o relevance of androgen signaling. ColAR-MC3T3 cultures were treated with DHT and the effects on osteoblast apoptosis were assessed by three independent assays: accumulation of cytoplasmic mono- and oligonucleosomes, caspase-3 activity using DEVD cleavage, or changes in mitochondrial membrane potential. In proliferating cultures, apoptosis was induced by treatment with the topoisomerase II inhibitor etoposide (50μM; 18h). Continuous treatment with DHT (10^{-8}M DHT; 5 days) enhanced apoptosis over two-fold (P<0.001), but as expected similar treatment with 17ß-estradiol (E2) inhibited apoptosis. In highly confluent osteocytic cultures (day 29) apoptosis was induced by serum withdrawal. In osteocytic cultures continuous DHT treatment also enhanced (P<0.001) while E2 inhibited apoptosis. The involvement of apoptotic regulators bcl-2 (antiapoptotic) and bax (proapoptotic) was characterized by qRT-PCR and Western analysis. Bcl-2 mRNA levels were unchanged and Bax was modestly elevated with DHT treatment by qRT-PCR. The ratio of Bax to Bcl-2 was increased by Western analysis. Overexpression of bcl-2 or knockdown of bax expression with siRNA abrogated the effect of DHT to enhance osteoblast apoptosis. The physiologic relevance of androgen regulation of osteoblast apoptosis was evaluated in vivo in calvaria from male AR-transgenic mice. TUNEL staining demonstrated increased apoptosis in areas of new bone growth and in osteocytes. These results are consistent with the hypothesis that apoptosis is important during bone growth to make room for new bone. Collectively, these findings demonstrate that androgen signaling through the AR in bone directly influences osteoblast survival and offers valuable insight into the role of androgen in bone homeostasis.

Appendix 4
*Wiren KM, Toombs A, Kasparcova V, Harada S, Zhang X-W: Androgens inhibit osteoclast activity in AR-transgenic mice through reductions in RANKL/OPG ratio. (Abstract #F202/SA202) J Bone Miner Res, 2004
           *Selected for the Plenary Poster Session award

Androgens Inhibit Osteoclast Activity In AR-Transgenic Mice Through Reductions In RANKL/OPG Ratio

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Our appreciation of the physiological functions of androgens has been changing. We recently characterized transgenic mice with AR overexpression in the osteoblast lineage under control of type I collagen promoter (col3.6 AR-transgenic mice), demonstrating proof of principle for the specific importance of androgen transactivation of AR directly in bone. Static and dynamic histomorphometric analysis in male AR-tg mice revealed site-specific effects on bone formation consistent with an anti-resorptive response. Characterization of gene expression by quantitative RT-PCR (qRT-PCR) in RNA isolated from tibial mid-diaphysis demonstrated reduced expression for osteoclastic genes including cathepsin K, RANKL and TRAP, while OPG expression was increased in males. Increased OPG serum levels were also observed. Since these results suggest osteoblast lineage-mediated inhibition of osteoclast activity, we characterized androgen control of osteoclastogenesis using a coculture system with monocytic RAW 264.7 cells as osteoclast precursors and col3.6 AR-MC3T3 cultures as a model of enhanced androgen responsiveness for osteoblasts. Cultures were treated with vehicle, 10^{-8}M DHT or soluble recombinant RANKL (50 ng/ml) alone, or DHT and sRANKL in combination. TRAP+ multinucleated cells containing 3 or more nuclei were considered formation of osteoclast-like cells. After 7 days, DHT inhibited osteoclast formation over 50% ($P<0.001$). The effects of DHT were abrogated with coadministration of sRANKL. The response in coculture with control ß-gal MC3T3 (no AR overexpression) demonstrated the same pattern but with reduced androgen efficacy. To evaluate the stage of osteoblast differentiation mediating androgen inhibition of osteoclastogenesis, OPG and RANKL mRNA was assayed using qRT-PCR at day 5 (proliferating preosteoblasts) and day 25 (osteocytic) colAR-MC3T3 cells, and with the osteocyte-like cell line MLO-Y4 (transiently transfected with an AR expression construct). Consistent with the effects in vivo, DHT inhibited RANKL but stimulated OPG gene expression in MLO-Y4 cells and day 25 colAR-MC3T3 osteoblastic cells. In contrast, in day 5 proliferating osteoblasts RANKL expression was undetectable while OPG expression was inhibited. These results demonstrate that inhibition of osteoclastogenesis occurs primarily by suppression of RANKL and/or elevation of OPG in mature osteoblasts. Collectively, these findings demonstrate that androgen signaling through the AR in bone directly influences osteoblast-osteoclast signaling and offers valuable insight into the role of androgen in bone homeostasis.

Appendix 5
*Wiren KM, Toombs AS, Matsumoto AM, Zhang X-W: Androgen receptor transgenic mice have decreased body fat, reduced adipogenesis and an increased osteoblast differentiation program. (Abstract #1198) J Bone Miner Res, 2005
     *Selected for Oral Presentation

Androgen Receptor Transgenic Mice Have Decreased Body Fat, Reduced Adipogenesis And An Increased Osteoblast Differentiation Program
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The direct effects of androgen signaling on bone are demonstrated in transgenic mice with enhanced androgen sensitivity, using androgen receptor (AR) under the control of the 3.6-kb α1(I)-collagen promoter (AR-tg). Male AR-tg mice show a complex anabolic bone phenotype with no change in circulating steroid levels. Since androgens are known to influence body composition, we hypothesized that targeted overexpression of AR in bone marrow stromal cells (MSCs) could alter lineage commitment to bone, fat and muscle. Body composition was analyzed by DXA, fat depot weights and adipogenesis in response to 5α-dihydrotestosterone (DHT) in MSC cultures. Gene expression was evaluated with qRT-PCR analysis. Male AR-tg 6 month old mice showed significantly increased BMC/body weight ($P < 0.01$), percentage lean mass ($P < 0.001$) but reduced percentage fat mass ($P < 0.001$) by DXA. Fat pad dissection from male AR-tg versus littermate controls showed significantly reduced white adipose tissue in visceral ($P < 0.05$) and gonadal ($P < 0.01$) fat, with no effect in brown fat. AR-tg expression was targeted to bone, with the highest expression in calvaria but ~100-1000 fold lower in mature fat, muscle, skin, kidney, liver, and spleen. Fresh marrow isolates showed AR-tg expression; only males showed reduced PPARgamma expression. Since androgen is hypothesized to influence body composition through modulation of MSC lineage allocation, we investigated adipogenic differentiation in MSC cultures. MSC were grown to confluence, then switched to adipogenic media. AR-tg MSC DHT-treated cultures showed dramatic inhibition of adipogenesis assessed by Oil Red O staining, with greater inhibition of PPARgamma expression versus controls. M2-10B4 stromal cell cultures also showed reduced Oil Red O after DHT treatment. Conversely, osteoblast differentiation was enhanced assessed by alkaline phosphatase activity. Thus, male AR-tg mice have decreased adiposity, increased lean mass and increased osteoblast differentiation due to enhanced androgen signaling without systemic androgen treatment. These results are consistent with late-onset obesity observed in global AR null animals and provide *in vivo* confirmation of the hypothesis that AR in MSCs directs lineage allocation. Further analysis will be important to define the distinct contributions of androgens to the maintenance of healthy body composition, and may identify regulatory pathways responsible for the deleterious consequences of hypogonadism and aging on central fat accumulation, muscle weakness and declining bone mass.

Appendix 6

CURRICULUM VITAE
Kristine May Wiren, Ph.D.

WORK ADDRESS: Department of Veterans Affairs Medical Center
Research Service (P3-R&D39)
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Portland, OR 97239
phone: 503.220.8262, ext. 56592 or 56595
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503.671.9880

EDUCATION:

1978 – 1984	PhD (Biomedical Science), 1985, University of Connecticut Health Center, Farmington (Thesis advisor Dr. Gideon Rodan, Chairman)
1975 – 1977	Portland State University, Portland, Oregon
1970 – 1975	BS (General Science) with High Scholarship, 1974, Oregon State University, Corvallis

ACADEMIC POSITIONS:

2001 – present	Associate Professor, Departments of Medicine and Behavioral Neuroscience, Oregon Health Sciences University, Portland
1994 – 2001	Assistant Professor, Department of Behavioral Neuroscience, Oregon Health Sciences University, Portland
1990 – present	Research Biologist, Research Service, Portland VA Medical Center, Portland
1990 – 2001	Assistant Professor, Department of Cell and Developmental Biology, Adjunct Assistant Professor, Department of Medicine, Oregon Health Sciences University, Portland
1988 – 1990	Research Molecular Biologist, Geriatric Research, Education and Clinical Center (GRECC), American Lake VA Medical Center, Tacoma, WA
1988 – 1990	Research Associate, Department of Medicine, University of Washington, Seattle
1984 – 1988	Research Fellow in Medicine, Harvard Medical School, and Endocrine Unit, Massachusetts General Hospital, Boston (Preceptor Dr. Henry Kronenberg, Chairman)

AWARDS AND HONORS:

2000 IBMS/Bone Best Paper Award Nomination in the Basic Research Category
Teaching Excellence Award for the Basic Science Graduate Program OHSU, 1998 – 1999
Young Investigator Award - Second International Conference on the Chemistry and Biology
    of Mineralized Tissues, 1984
University of Connecticut Travel Awards, 1981, 1982, 1983
University of Connecticut - Graduate Student Scientific Day abstract selected for oral
    presentation and publication in Threshold Magazine
University of Connecticut Predoctoral Fellowships, 1978 – 1982
National Honor Society of Phi Kappa Phi, 1974

RESEARCH INTERESTS:

One broad aim in my laboratory is the characterization of mechanisms underlying alterations in skeletal function mediated by steroid hormones, in particular through androgen action. Generally, steroid alterations are brought about at the molecular level by specific regulation of gene transcription, controlled by the steroid hormone receptors after ligand binding. My laboratory studies focus on characterizing the sequelae to estrogen or androgen exposure in osteoblasts or stromal lineage cells that can form bone, cartilage, fat and muscle. The *in vivo* consequence of overexpression of androgen receptor in the osteoblast lineage is being evaluated in two AR-transgenic mouse models. Another model system that is employed uses normal osteoblasts grown *in vitro* that eventually develop a mineralized bone matrix. These cultures are exposed to sex steroids, and regulation of osteoblast gene expression during normal proliferation and differentiation is characterized at the RNA and protein level. In a broad sense, studies in my laboratory have the long-term goal of developing a better understanding of mechanisms that underlie the beneficial effects of sex steroids on the skeleton and to influence body composition.

A second area of research in my laboratory is characterization of molecular mechanisms underlying neuroadaptation to chronic ethanol exposure, focusing on changes in gene expression in various brain regions observed during ethanol exposure/withdrawal in both genders. Current studies are carried out in males and females using both seizure-prone or seizure-resistant selected lines of mice and in inbred strains to identify sequences that are persistently regulated after chronic exposure and withdrawal. The laboratory is using a molecular biological approach that includes microarray analyses and real-time RT-PCR to identify and characterize regulated transcripts. Sex-specific responses in gene expression indicate increased potential for brain damage in females that may reflect changes in circulating sex steroids. The goal of these studies is to better understand how changes in gene expression may underlie physical dependence and the detrimental consequences that result from withdrawal from ethanol or other drugs of abuse.

GRANT SUPPORT (annual direct costs):

Current funding:

Department of Defense:

2004 – 2008: Principal Investigator, DOD/USAMRMC (PR043014), "Enhanced androgen signaling with androgen receptor overexpression in the osteoblast lineage controls skeletal turnover, matrix quality and bone architecture"; $355,130 (annual direct costs)

NIH:

2006 – 2011: Principal Investigator, NIH/NIDDK (R01 DK067541) Research Grant, "Androgen action in bone: overexpression of AR"; $293,015 (annual direct costs)

2006 – 2011: Co-Investigator (PI: M. Bliziotes, MD), NIH/NIAMS (R01 AR052018) Research Grant, "The function of neurotransmitters in bone biology"; costs for Wiren component $35,000 (annual direct costs)

2004 – 2008: Co-Investigator (PI: R. Jones, PhD), NIH/NINDS (R01 NS039122) Research Grant, "Mechanisms of disease progression in EAE"; costs for Wiren $15,000 (annual direct costs)

Pending funding:

2006 – 2010: Principal Investigator, VA Merit Review, "Neurotoxic effects of alcohol in females: role of androgen"; $268,800 (annual direct costs)

2006 – 2011: Principal Investigator, NIH/NIAMS-NIA (R01 AG028912), "Androgen and aging: adipogenesis through altered bone marrow lineage allocation"; $250,000 (annual direct costs)

2006 – 2011: Co-Principal Investigator (PI: D. Finn, PhD), NIH/NIAAA Research Grant (R01 AA12439), "Neurosteroid modulation of ethanol withdrawal severity"; costs for Wiren component $6,000 (annual direct costs)

Previous funding:

Department of Veterans Affairs:

1994 – 2005: Principal Investigator, VA Merit Review "Molecular mechanisms of androgen regulation of osteoblast function"; $168,000

1990 – 1994: Principal Investigator, VA Merit Review "Estrogen regulation of the osteoblast - analysis by molecular cloning", $100,000

1989 – 1990: Principal Investigator, VA Research Advisory Group Grant "Estrogen regulation of the osteoblast - analysis by molecular cloning"; $40,000

NIH:

2001 – 2005: Principal Investigator, NIAAA Research Grant (R01 AA13194), "Persistent changes in brain expression after ethanol withdrawal"; $150,000 (annual direct costs)

2000 – 2001: Principal Investigator, Alcohol Research Center Grant (P50 AA10760) Pilot Grant, "Regulation of kappa opioid receptor activation: potential role in mediating the motivational effects of ethanol"; $25,000

2000 – 2005: Co-Principal Investigator (PI: D. Finn, PhD), Alcohol Research Center Grant (P50 AA10760) Component #5, "Ethanol withdrawal and neuroactive steroids"; costs for Wiren $6,000 (annual direct costs)

1998 – 2004: Co-Investigator (PI: J. Belknap, PhD), NIAAA Research Grant (R01 AA06243), "Alcohol predisposition-comparative withdrawal syndromes"; costs for Wiren component $63,000

1998 – 2001: Co-Investigator (PI: M. Bliziotes, MD), NIDDK Research Grant (R01 DK54415), "G protein-coupled receptor kinases in bone biology"; costs for Wiren component $30,000

1995 – 2002: Principal Investigator, Alcohol Research Center Grant (P50 AA10760) Component #9, "Genetics of neuroadaptation to ethanol"; $60,000

1994 – 1995: Principal Investigator, Gerlinger Research Foundation, "Relationship between collagen synthesis and the osteoblast phenotype"; $10,400

1993 – 1996: Co-Investigator (PI: E. Orwoll, MD), NIDDK Research Grant (R01 DK46668), "The androgen receptor in osteoblasts"; $78,000

1991 – 1992: Principal Investigator, Medical Research Foundation of Oregon, "Molecular cloning analysis of androgen regulation of gene expression in osteoblasts"; $25,000

1986 – 1987: Principal Investigator, NRSA Individual Postdoctoral Fellowship Grant "Molecular Genetics of Parathyroid Hormone Secretion"

Industry:

2003 – 2004: Principal Investigator, Merck & Co., Inc., "Overexpression of androgen receptor in osteoblasts"; $17,200 (annual direct costs)

Sponsored fellowships:

1999 – 2002: NRSA Individual Predoctoral Fellowship Grant, sponsor for Amy Beadles-Bohling, "Analysis of the kappa-opioid system in ethanol withdrawal"

2000 – 2001: Predoctoral Tartar Trust, sponsor for Amy Beadles-Bohling, "Analysis of the kappa-opioid system in ethanol withdrawal"
2000 – 2001: Predoctoral Tartar Trust, sponsor for Tarra Guptaa, "Involvement of mineralocorticoid receptor in withdrawal severity after repeated ethanol withdrawal"
1996 – 1997: NRSA Individual Predoctoral Fellowship Grant, sponsor for Gwen Schafer, "Effects of ethanol exposure on gene expression in mice"

PROFESSIONAL ACTIVITES:

Administrative and Academic Committees:

2004 – present   Faculty Council, School of Medicine, OHSU
2003 – 2004   Faculty Senate, Internet Filter Committee
2002, 2003   Faculty Senate, School of Medicine Excellence Award Subcommittee
2001 – 2003:   Faculty Senate, Research Subcommittee, School of Medicine, OHSU
2001 – 2003:   Research and Development Committee, Department of Veterans Affairs
2000 – 2003:   Faculty Senate, School of Medicine, OHSU
1999 – present: Chair, Awards Committee, Department of Behavioral Neuroscience, OHSU
1997 – present: Graduate Student Recruitment Committee, Department of Behavioral Neuroscience, OHSU
1994 – 2000:   Doctoral Studies Committee, Department of Behavioral Neuroscience, OHSU
1998 – present: Graduate Student Admissions Committee, Department of Behavioral Neuroscience, OHSU (1998, 2000, 2005)
1999 – 2000:   Project Review Committee, Department of Veterans Affairs
1996   Grievance Panel, OHSU
1991 – 1995:   Chair, Research Biosafety Subcommittee, Department of Veterans Affairs
1991 – 1995:   Hospital Safety and Risk Management Committee, Department of Veterans Affairs
1989 – 1990:   Institutional Review Board, Madigan Army Medical Center

Professional Society Membership:

American Society for Bone and Mineral Research (ASBMR); Research Society on Alcoholism (RSA);The Endocrine Society

Grant Review Service:

NIDDK Study Section, Skeletal Biology Structure & Regeneration (formerly ORTH) 2000 – 2004 *ad hoc*
NIAAA Biomedical Research Review Subcommittee AA1 2003, 2004, 2005 ad hoc
NIAAA Special Emphasis Panel ZAA1 CC(01), 2003
NIAAA Special Emphasis Panel ZAA1 CC(12), 2003
NIAAA Reverse Site Visit ZAA1 AA(57), 2003
NIAAA Special Emphasis Panel ZAA1 CC(15), 2002
VA Merit Review Grants
Medical Research Council (MRC) Grant, Canada

Editorial Service (*ad hoc* reviewer):

BioTechniques; Endocrinology; Epilepsia; Functional & Integrative Genomics; Brain Research (and Developmental, Molecular); Biological Psychiatry; Alcohol; Neurochemistry International; Pharmacology, Biochemistry and Behavior; Journal of Clinical Endocrinology & Metabolism; Journal of Orthopaedic Research; Journal of Bone and Mineral Research; Journal of Bone and Mineral Research (Invited book review); GH and IGF Research Journal; Asian Journal of Andrology.

INVITED PRESENTATIONS / SYMPOSIA CHAIR / SOCIETY FUNCTIONS:

International:

2004:	Invited Speaker "Effects of chronic alcohol exposure and withdrawal on CNS gene expression", RSA 27th Annual Meeting, Vancouver, Canada

National:

2005 – 2006	Category Chairperson for Osteoblasts: other; 2006 ASBMR Program Committee
2005 – 2008	ASBMR Science Policy Committee
2005:	Co-Organizer/Co-Chair Symposia: "Impact of sex: determination of alcohol neuroadaptation and reinforcement"; RSA 28th Annual Meeting
2005:	Invited Speaker, RSA 28th Annual Meeting, "The importance of gender in determining expression differences in mouse lines selected for chronic ethanol withdrawal severity"
2005:	Scientific Program Committee; ASBMR 27th Annual Meeting
2004:	Scientific Program Committee; ASBMR 26th Annual Meeting
2004:	Invited Speaker Analysis of Gene Expression: A Real-Time PCR Technical Forum (Sponsored by Bio-Rad); Joel Hashimoto and Kristine Wiren, "Comparison of RiboGreen and 18S Real-Time PCR for normalizing Real-Time RT-PCR"
2004:	Invited Speaker OHSU NIAAA/NIDA Training Grant Retreat, Portland OR
2003:	Co-Chair "Osteoblasts III"; ASBMR 25th Annual Meeting
2002:	Co-Chair "Steroid Hormones I"; ASBMR 24th Annual Meeting
2001:	Invited Speaker Mt. Sinai School of Medicine, "Distinct Modulation of Osteoblast Function by Estrogen or Androgen"
1999:	Invited Speaker OHSU Cell and Developmental Biology seminar series, "Distinct Modulation of IGF action by either Estrogen or Androgen in Bone Cells"
1998:	Co-Chair Endocrine Society 80th Annual Meeting, "PTH-Calcium-Vitamin D-Bone II"
1997:	Invited Speaker OHSU Neuroscience Graduate Program seminar, "Regulation of gene expression by ethanol"
1995:	Invited Speaker Merck, Sharpe & Dohme, "The androgen receptor in osteoblasts: expression and regulation"

TEACHING/TRAINING:

Courses:

1993 – present:	Participation/discussion in Department of Behavioral Neuroscience seminar series *Issues in Behavioral Neuroscience* BEHN 607*;* Co-organizer 2003; Topic Steroid Hormones and Withdrawal
1995 – present:	Course co-coordinator and primary lecturer; BEHN 619 *Molecular Strategies in Behavioral Research* (3 credits) presented bi-annually (sometimes annually) for the Departments of Behavioral Neuroscience, Physiology/Pharmacology, Neuroscience graduate

	students, and medical and dental students, covering basic molecular biology research methods
1997 – present:	Lecturer in the *Oregon Human Investigations Program* (HIP) course (previously *Techniques and Topics in Molecular Biological Research)* presented annually to medical residents & fellows (Dave Brandon PhD, course developer), one 1½ hour lecture covering basic molecular biology methods including gel electrophoresis, restriction digests and cDNA cloning
1999 – 2004:	Lecturer in *Molecular Biology in Neurology* presented to medical residents & fellows, three-one hour lectures covering basic molecular biology and control of gene expression
2000 – present	Presenter; BCMB 605 *Signal Transduction Journal* Club
2003 – present	Lecturer in BEHN 617 *Neurochemical Basis of Addiction.* Topic: Nuclear Receptors: Mechanisms and Action presented biannually.

CME Presentations:

2002	Material for presentation with Amy Beadles-Bohling; *Clinician Training* for the Northwest Portland Area Indian Health Board "Practical applications of science-based research regarding DNA synthesis and repair"

Teaching Activities:

1993 – present:	Training Grant Faculty Member: NIDA Training Grant; "Biological bases of drug-seeking behavior", Department of Behavioral Neuroscience, OHSU
1993 – present:	Training Grant Faculty Member: NIAAA Training Grant; "Biological bases of alcoholism", Department of Behavioral Neuroscience, OHSU

Students/Postdoctoral Fellows/Faculty Supervised:

Post-doctoral preceptor:	Theresa Madden, DDS, PhD, 1995 – 1996 (presently an Associate Professor in the Department of Periodontology, OHSU)
	Anthony Semirale, PhD, 2005 – present
Pre-doctoral preceptor:	Gwen Schafer, Department of Behavioral Neuroscience, 1995 – 1998, (PhD 1998, presently at the Mental Health Research Institute, University of Michigan)
	Amy Beadles-Bohling, Neuroscience Graduate Program, 1997 – 2004 (PhD 2004, presently at OHSU in post-doctoral training)
	Tarra Guptaa, Department of Behavioral Neuroscience, 1998 – 2001
Second year project:	Rebecca Hammond, Department of Behavioral Neuroscience; co-advisor 2004 – 2005
Research rotations:	Amy Beadles-Bohling 1997
	Sarah Coste 1996 – 1997
	Tarra Guptaa, 1998
	Tara Macey, 1999
	Christopher Kliethermes, 2001
	Deaunne Denmark, 2005
MD Fellow training:	Bruce Boston, MD 1992 – 1993
	Linda Lester, MD 1995

Kathy Pillotte, MD 1996 – 1998
Laurie Vessely, MD 1999 – 2000

Thesis committee service:
MPh: Charlene Bryan, Masters of public health, degree 1999
PhD: Pam Metten, Doctoral dissertation, degree 1996
              Sarah Coste, Doctoral dissertation, degree 1997
              Gwen Schafer, Doctoral dissertation, degree 1998
              Stephen Boehm, Doctoral dissertation, degree 2002
              Alison Atkins, Doctoral dissertation, degree 2004
              Christopher Kliethermes, Doctoral dissertation in progress
              Sarah Holstein, Doctoral dissertation in progress

Science Outreach:
1995 – present: Matt Rodland (undergraduate) Summer Intern, 1995
                  Heather Burpee (undergraduate) Summer Intern, 1997
                  Andrew Buckman (high school student) Summer Intern, 1997
                  Ian Schriener (high school student) Summer Intern, 1998
                  Maya Narayanan (high school student) School year, 1999
                  David Schlesinger (high school student) School year, 2000
                  Jessi Li (high school student) Summer Intern, 2001
                  E. Lutomia Kuto (high school) Summer Research Minority Apprenticeship
                      Program, 2001
                  Sophia Fang (undergraduate) Summer Intern, 2003
                  Alexandria Luftig (high school senior) VA Summer Fellowship Program,
                      2003
                  Gus Lawrence (undergraduate) Summer Intern, 2005
1996 – 2005:   Judge at Graduate Student Research Forum
1998 – 2001:   Lab support in AWSEM (Advocates for Women in Science, Engineering
                      and Mathematics) course on DNA (taught by Amy Beadles-Bohling)
1998 – 2000:   Lab support in Saturday Academy course on DNA (taught by Amy
                      Beadles-Bohling)
1997, 2000   Public outreach, Portland Alcohol Research Center, VAMC Research Day
                      tours
1996 – 1997:   Moderator at "Sym Bowl" computer modeling competition for high school
                      students
2004   Special Award Judge (Endocrine Society) for Intel International Science
                      and Engineering Fair (ISEF)

BIBLIOGRAPHY:

Peer-reviewed publications:

Kronenberg HM, Igarashi T, Freeman MW, Okazaki T, Brand SJ, **Wiren KM**, Potts JT Jr:
    Structure and expression of the human parathyroid hormone gene. Rec Prog Horm Res
    42:641-663, 1986

Wiren KM, Freeman MW, Potts JT, Kronenberg HM: Preproparathyroid hormone: a model
    for analyzing the secretory pathway. Ann NY Acad Sci 493:43-49, 1987

Freeman MW, **Wiren KM**, Rapaport A, Lazar M, Potts JT, Kronenberg HM: Consequences
    of amino-terminal deletions of preproparathyroid hormone signal sequence. Mol
    Endocrinol 1:628-638, 1987

Wiren KM, Potts JT Jr, Kronenberg HM: Importance of the propeptide sequence of human preproparathyroid hormone for signal sequence function. J Biol Chem 263:19771-19777, 1988

Wiren KM, Ivashkiv L, Ma P, Freeman MW, Potts JT Jr, Kronenberg HM: Mutations in signal sequence cleavage domain of preproparathyroid hormone alter protein translocation, signal sequence cleavage, and membrane-binding properties. Mol Endocrinol 3:240-250, 1989

Plymate SR, Loop S, Hoop RC, **Wiren KM**, Ostenson R, Hryb DJ, Rosner W: Effects of sex hormone binding globulin (SHBG) on human prostatic carcinoma. J Steroid Biochem Mol Biol 40:833-839, 1991

Baba H, Karaplis AC, **Wiren KM**, Keutmann HT, Kronenberg HM: Interaction of nascent preproparathyroid hormone molecules with microsomal membranes. J Bone Miner Res 7:199-206, 1992

Kuijper JL, **Wiren KM**, Mathies LD, Gray CL, Hagen FS: Functional cloning vectors for use in directional cDNA cloning using cohesive ends produced with T4 DNA polymerase. Gene 12:147-155, 1992

Klein RF, **Wiren KM**: Regulation of osteoblastic gene expression by lead. Endocrinology 132(6): 2531-2537, 1993

Birnbaum RS, **Wiren KM**: Changes in insulin-like growth factor binding protein expression and secretion during the proliferation, differentiation, and mineralization of primary cultures of rat osteoblasts. Endocrinology 235(1):223-230, 1994

Birnbaum RS, Bowsher RR, **Wiren KM**: Changes in insulin-like growth factor-I expression and secretion during the proliferation and differentiation of normal rat osteoblasts. J Endocrinology 144:251-259, 1995

Bliziotes M, Murtagh J, **Wiren KM**: Identification of the ß-adrenergic receptor kinase and ß-arrestin in rat bone. J Bone Miner Res 11(6):820-826, 1996

Wiren KM, Zhang X-W, Chang C, Keenan E, Orwoll E: Transcriptional up-regulation of human androgen receptor by androgen in bone cells. Endocrinology 138:2291-2300, 1997

Schafer GL, Crabbe JC, **Wiren KM**: Identification of neuroendocrine-specific protein as an ethanol-regulated gene using mRNA differential display. Mamm Genome 9:979-982, 1998

Wiren KM, Keenan E, Zhang X-W, Ramsey B, Orwoll E: Homologous androgen receptor upregulation in osteoblastic cells may be associated with enhanced functional androgen responsiveness. Endocrinology 140:3114-3124, 1999

Beadles-Bohling AS, Crabbe JC, **Wiren KM**: Elevated prodynorphin expression associated with ethanol withdrawal seizures. Neurochem Internat 37:463-472, 2000

Bliziotes M, Gunness M, Zhang X-W, Nissenson R, **Wiren K**: Reduced G protein-coupled receptor kinase 2 activity results in impairment of osteoblast function. Bone 27:367-373, 2000

Schafer GL, Crabbe JC, **Wiren KM**: Ethanol-regulated gene expression of neuroendocrine-specific protein in mice: brain region and genotype specificity. Brain Res 897(1-2):139-149, 2001

Billiard J, Umayahara Y, **Wiren K**, Centrella M, McCarthy TL, Rotwein P: Regulated nuclear – cytoplasmic localization of CCAAT/enhancer binding protein delta in osteoblasts. J Biol Chem 276(18):15354-15361, 2001

Bliziotes M, Eshleman A, Zhang X-W, **Wiren KM:** Neurotransmitter action in osteoblasts: expression of a functional system for serotonin activation and reuptake. Bone 29(5):477-486, 2001

Bliziotes M, Gunness M, Eshleman A, **Wiren K**: The role of dopamine and serotonin in regulating bone mass and strength: Studies on dopamine and serotonin transporter null mice. J Musculoskel Neuron Interact 2:291-295, 2002

Wiren KM, Evans AC, Zhang XW: Osteoblast differentiation influences androgen and estrogen receptor α and ß expression. J Endocrinol 175(3): 683-694, 2002

Finn DA, Ford MM, **Wiren KM**, Roselli CE, Crabbe JC: The role of pregnane neurosteroids in ethanol withdrawal: behavioral genetic approaches. Pharmacol Ther 101(2):91-112, 2004

Zhang X-W, Toombs AR, **Wiren KM**: Androgen inhibition of MAP kinase pathway and Elk-1 activation in proliferating osteoblasts. J Mol Endocinol 32:209–226, 2004

Wiren KM, Zhang X-W, Toombs AR, Gentile MA, Kasparcova V, Harada S-I, Jepsen KJ: Targeted overexpression of androgen receptor in osteoblasts: unexpected complex bone phenotype in growing animals. Endocrinology 145:3507-3522, 2004.

Hashimoto J, Beadles-Bohling A, **Wiren KM**: Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis. BioTechniques 36:54-60, 2004

McBride WJ, Kerns RT, Rodd ZA, Strother WN, Edenberg HJ, Hashimoto JG, **Wiren KM**, Miles MF: Alcohol effects on central nervous system gene expression in genetic animal models. Alcohol Clin Exp Res 29(2):167-175, 2005

Beadles-Bohling A, **Wiren KM**: Altered kappa opioid receptor system expression in distinct brain regions of a genetic model of enhanced ethanol withdrawal severity. Brain Res 1046(1-2):77-89, 2005

Warden S, Bliziotes M, **Wiren K,** Eshleman A, Turner C: Neural regulation of bone and the skeletal effects of serotonin (5-hydroxytryptamine). Mol Cell Endocrinol 242(1-2):1-9, 2005

Wiren KM: Androgen action and bone growth: it's location, location, location. Curr Opin Pharmacol 5:626-632*,* 2005

Wiren KM, Toombs AR, Semirale AA, Zhang X-W: Apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio. Bone *in press*, 2006

Beadles-Bohling A, **Wiren KM**: Anticonvulsive effects of kappa opioid receptor modulation in an animal model of ethanol withdrawal. Genes Brain Behav *in press*, 2006

Wiren KM, Hashimoto JG, Alele PE, Devaud LL, Price KL, Middaugh LD, Grant KA, Finn DA: Impact of sex: Determination of alcohol neuroadaptation and reinforcement. Alcohol Clin Exp Res *in press*, 2006

In preparation publications:

Hashimoto J, **Wiren KM:** Neurotoxic consequences of chronic alcohol withdrawal: microarray analyses reveal importance of gender over withdrawal severity phenotype. J Neuroscience *in preparation*, 2006

Ford MM, Hashimoto JG, Samson HH, Finn DA, **Wiren KM**: Hypothalamic neuroadaptation during chronic ethanol self-administration in female rats. *Manuscript in preparation*, 2006

Finn D**, Wiren KM**, Long S, Tanchuck M, Hashimoto J, Roselli C: Ethanol dependence and withdrawal differentially alters allopregnanolone levels and 5α-reductase-1 enzyme activity and gene expression in Withdrawal Seizure–prone and –resistant mice. *Manuscript in preparation*, 2006

Chapters and invited reviews:

Rodan GA, Majeska RJ, **Wiren KM**, Rodan SB: Expression of hormonal effects in osteosarcoma osteoblastic cells. In: Proceedings of the VIII International Conference on Calcium Regulating Hormones, Endocrine Control of Bone and Calcium Metabolism,

Vol. 8A (DV Cohn, T Fujita, JT Potts Jr, RV Talmage, Eds), Amsterdam, Elsevier Science Publishers, pp. 117-124, 1984

Wiren KM, Rodan GA: Dissociation between the effects of glucocorticoids on cell spreading and on phenotypic expression. In: The Chemistry and Biology of Mineralized Tissues, (WT Butter, Ed), Ebsco media, Inc., Birmingham AL, pp. 320-325, 1985

Kronenberg HM, **Wiren KM**, Freeman MW, Potts JT Jr: Preproparathyroid hormone: use of mutants to study secretion. In: Proceedings of the IX International Conference on Calcium Regulating Hormones, Calcium Regulation and Bone Metabolism (Basic and Clinical Aspects), Vol 9 (DV Cohn, TJ Martin, PJ Meunier, Eds) Amsterdam, Excerpta Medica, pp 773-777, 1987

Potts JT Jr, Nussbaum S, **Wiren KM**, Freeman M, Igarashi T, Okasaki T, Zajak J, Abou-Samra AB, Bringhurst R, Segre G, Kronenberg H: Chemistry, molecular biology and mode of action of parathyroid hormone. In: 1st International Conference on New Actions of Parathyroid Hormone, October 26-31, Kobe, Japan, pp. 26-31, 1987

Keith LD, Roberts AJ, **Wiren K**, Crabbe JC: Corticosteroid-ethanol interactions. In: Stress, Gender, and Alcohol-seeking Behavior, (WA Hunt and S Zakhari, Eds), NIAAA Research Monograph No. 29, NIH publication No. 95-3893, pp. 181-196, 1995

Gallaher E, Keith LD, **Wiren KM**: Dynamic properties of antisense nucleotides and their impact on mRNA and receptor protein. Western Multiconference Society for Computer Simulation, 1996

Orwoll ES, **Wiren KM**: Androgens and bone: basic aspects. In: Osteoporosis in men, (ES Orwoll, Ed), Academic Press, San Diego, CA, pp. 211-245, 1999

Wiren KM: Invited book review on: The Aging Skeleton, (CJ Rosen, J Glowacki, JP Bilezikian, Eds), Academic Press, San Diego, CA., in J Bone Miner Res, 15:1640, 2000

Wiren KM, Orwoll ES: Skeletal Biology of Androgens. In: Osteoporosis, (R Marcus, Ed), Academic Press, San Diego, CA, pp. 339-359, 2001

Wiren KM, Orwoll ES: Androgens: receptor expression and steroid action in bone. In: Principles of Bone Biology, (JP Bilezikian, LG Raisz and GA Rodan, Eds), Academic Press, San Diego, CA, pp. 757-772, 2002

Wiren KM: Androgen Action in Bone: Basic Cellular and Molecular Aspects. In: Osteoporosis: Pathophysiology and Clincial Management, (MM Bliziotes and ES Orwoll, Eds), The Humana Press, Inc., Totowa, NJ, pp. 349-373, 2002

Crabbe JC, **Wiren KM**: Molecular and Genetic Bases of the Addictions. In: The Molecular and Genetic Basis Of Neurologic and Psychiatric Disease, (R Rosenberg, S Prusiner, S DiMauro, R Barchi and E Nestler, Eds), Butterworth-Heinemann Medical Publishing, Woburn, MA, pp. 779-789, 2003

Wiren KM, Orwoll ES: Skeletal Biology of Androgens. In: Osteoporosis, (R Marcus, Ed), Academic Press, San Diego, CA, *in press*, 2006

Abstracts:

Wiren KM, Rodan GA: Glucocorticoid effects on cell spreading and fibronectin production by cultured osteoblast-like cells. East Coast Connective Tissue Society, 1983

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